THE IMMUNOLOGICAL SIGNIFICANCE OF COLOSTRUM

III. Intranuclear Bodies in Renal Disease of Calves

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Plates 9 and 10

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The urinary organs of young calves are vulnerable, and pathological conditions readily appear in an abnormal environment. Of these the so-called spotted kidney has been discussed in an earlier paper.[1] In this condition evidence points to a sublethal infection with *B. coli*. The process involves both organs alike. The multiple white foci of infiltrated cells are situated in the cortex and are embedded in normal tissue. The process ends either in a reabsorption of the exudate cells or in sclerosis whereby the enclosed functional structures are destroyed. The urinary secretion contains only a trace of protein and the lesion is not recognized until the animal is killed. The kidneys are as a rule somewhat heavier than the normal organ.

Another pathological condition expresses itself in large, uniformly white kidneys symmetrically involved. The lesions of the spotted kidney may be present also. Cases of the white kidney were observed several times in calves reared in the usual way about 10 years ago. Latterly experiments involving the withholding of colostrum and the feeding of serum in its place have brought a few to the surface. Before discussing the probable nature and etiology of the condition a few cases will be presented. To begin with, a case will be described in which the presence of intranuclear bodies was associated with nearly normal organs.

Calf 1532.[2] Holstein male born Jan. 11, 1929. Received in 3 consecutive doses, up to the 13th hour, 1400 cc. of cow serum mixed with cow's milk. 700 cc. of the

[1] Smith, T., *J. Exp. Med.*, 1925, **41**, 413.

[2] This calf is tabulated among others in *J. Exp. Med.*, 1930, **51**, 488.

serum came from untreated cows and 700 cc. from a cow treated with *B. coli* filtrates (D) and found only feebly protective for guinea pigs.[3] The serum had been stored for nearly 2 years. No significant amount of specific protective antibodies had thus been fed to this animal.

During the 2 months of life there were no disturbances except a brief period of liquid stools during the 2nd day. The urine following the heat test became faintly clouded throughout but there was no measurable deposit[4] of coagulum except on one occasion when it was 1.3 per cent. The gain in weight was normal, also the temperature curve. It was killed by a blow and severing neck vessels when 2 months and 8 days old. The following represent the few deviations from the normal found at autopsy.

The lymphoid tissue of the small intestine was below the normal in quantity but the thymus was normal in weight (300 grams). In the upper portion of the duodenum the mucosa was dotted with roundish elevations which were later recognized in sections as a lymphocyte infiltration of the villi. In the cecum the veins of the submucosa were marked by pigment deposits. The same condition extended 15 or 17 cm. below the ileocecal valve. The free border of the valve was deeply pigmented. In the upper colon were two areas showing numerous vague whitish spots 1–2 mm. in diameter. In sections, these spots were shown to be lymphoid follicles in submucosa. They were pear-shaped and the tapering portion extended into the mucosa between the tubules. A small number had reached the surface in the form of cylindrical plugs. Over them the surface epithelium had disappeared.

The kidneys were about 1⅛ times the average weight at this age (192 and 200 grams, respectively, with capsule stripped off). On section, faint pale radial striations could be detected in cortex. In frozen sections of the fresh tissue, the condition shown in Fig. 1 presented itself. In the columns of Ferrein the epithelium of the tubules contained irregular foreign bodies all located within the nuclei. Some were in cells of the convoluted tubules. They appeared as irregular agglomerations of 2μ discs, some 4 to 6 consolidated into a straight or bent line. The epithelium was not distorted or distended as yet. In scrapings of the fresh (unfrozen) tissue, the bodies were present, hence freezing had nothing to do with bringing them out. In sections of tissue fixed in Zenker and alcohol, they were not changed and were colored reddish in stains containing eosin. In general the kidney tissue appeared normal, with perhaps a very slight hyperplasia of the interstitial tissue. In Figs. 2 and 3 a thin film of scrapings of the cut surface stained in hematoxylin and eosin brings out the intranuclear position of these bodies. The cytoplasm of the epithelial cells has been broken up by the procedure and only the nuclei have retained their form.

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[3] For the method of testing protective power see *J. Exp. Med.*, 1930, **51**, 474. For a brief description of the cows furnishing serum see *J. Exp. Med.*, 1930, **51**, 483.

[4] For the rough estimation of protein coagulum in urine see *J. Exp. Med.*, 1930, **51**, 485.

This is the first and thus far the only animal in which the intraglobular bodies were found in mass under apparently still normal functioning of the kidneys and normal condition of the animal generally.

The following fatal case is perhaps typical of the uncomplicated syndrome. Death took place during the writer's absence and only the formalinized kidneys and Zenker-fixed portions were available.

Calf 1511.—Guernsey bull, born May 22, 1928. Fed 700 cc. serum of Cow C^5 (treated with *B. coli* mutant 1192_b) mixed with milk, in 3 doses as heretofore, the last dose when calf was about 13 hours old; thereafter only milk.

There were no disturbances of noteworthy extent during the 1st month. It was turned out into a paddock at this time and fed alfalfa in addition to the milk diet since it refused hay and grain. During the 2nd month it became unthrifty and constipated, the coat rough and abdomen distended. July 19, the calf was found down. It took milk very slowly. There was present tenesmus with passage of small amounts of feces. On July 20, it took a little milk early and was found dead at noon. The temperature of this calf taken twice daily was within normal range throughout if we except a slight rise on the 4th, the 27th–31st, and the 46th day.

Autopsy.[6] Mucous membranes pale. Subcutaneous tissues around buttocks and scrotum edematous, the fluid, blood-tinged. Both kidneys enclosed in a layer of edematous tissue. The left is also enclosed in a blood clot. The bladder and rectum are surrounded by edematous, blood-tinged tissue. On dorso-ventral tip of spleen a projecting, flattish, hemorrhagic elevation. When the kidneys are shelled out of the edematous envelopes, they are found larger than normal. The weights are for the left kidney 540 grams, for the right 460 grams. This is between 2 and 3 times the weight of normal kidneys at this age. Both appear uniformly white with numerous hemorrhagic points near surface from ½ to 2 mm. apart. On section, cortex whitish throughout with broad injected vessels radiating from the medullary boundary into both cortex and medulla.

The progress of the renal disease was reflected to a certain extent in the urine. Up to and including the 39th day of life there was a faint clouding when it was acidified and boiled. No further samples were tested until the day before death when the coagulum formed after heating was equivalent to 22.6 per cent in volume after 24 hours' sedimentation. Urine taken from bladder at the autopsy had the same volume of coagulum.

The kidneys hardened in Zenker's fluid and formalin were available for further study. Transections of the entire organ indicated some interference with the outflow since the calices were separated by a distinct space from the papillae. Stasis was further indicated by a general distention of the convoluted tubules

[5] See *J. Exp. Med.*, 1930, **51**, 484.

[6] By Dr. R. B. Little in the writer's absence.

beginning at or within the glomerulus. The cortical structures were also driven apart by edema. The interstitial connective tissue was slightly increased and this was moreover evidenced by occasional mitoses. Within the nuclei of the epithelium of the convoluted tubules were inclusions, in form disclike, about 2μ in diameter (Figs. 4 and 5). The number of discs in a nucleus varied up to 12. They were fused together in an irregular, longitudinal mass. As a result some cell nuclei were greatly distended. One measured 15μ by 20μ. Any definite geometrical form was not recognizable except the generally circular outline of the discs. The fused masses have sharp borders, homogeneous surface, and suggest crystals. The intranuclear situation is easily recognized except when the masses are largest. Even then the greatly distended nuclear membrane may be made out. They are found in the labyrinth and the columns of Ferrein and quite irregularly in these regions.

The hemorrhages are of two kinds, those starting from glomeruli and those about the loops of Henle. Scattered about are groups of hemin crystals. Hyaline casts are found in the smaller collecting tubules and Henle loops.

In the hardened tissue, in cross sections of some of the distended tubules, there are from one to all of the epithelial cells with intranuclear bodies. The involved cell usually projects well beyond its neighbors into the lumen. Rarely a group of two or three hypertrophied cells shed into the lumen nearly occludes it. In the medulla, occasional greatly dilated collecting tubules are encountered. In others, hyaline casts are present. Associated with these inclusions there is hypertrophy and some degeneration of cells in both primary and secondary convoluted tubules and moderate interstitial edema together with some cell infiltration probably monocytic. The striking feature is the general distension of tubules from Bowman's capsule down.

Some tests with acids and alkalies were made with frozen sections of the formalinized kidneys. Thus 4 per cent NaOH acting upon the sections for 18 hours caused no change in the inclusions. Similarly 5 per cent acetic acid failed to dissolve them, although they seemed a trifle smaller.

The following is another typical case.

Calf 1411.—Holstein male, born Jan. 12, 2.40 p.m. Receives 400 cc. serum of Cow D (*B. coli* filtrate) plus an equal quantity of milk. At 8 hours it receives 200 cc. and at 15 hours 100 cc., also with milk added; thereafter only milk. This animal passed through a relatively severe attack of liquid stools which lasted 5 days. Later, traces of protein appeared in the urine from time to time. On March 4, the urine was clouded with fine flocculi which were covered with masses of small diphtheroid rods. Cultures brought to light *B. pyogenes* and the specific cystitis bacillus.[7]

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[7] Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1926, **44**, 11.

March 23. Calf tends to lie down. Grits its teeth. Takes only a portion of the offered milk. Temperature about normal.

March 30. Calf has been growing weaker and taking only about one third the normal amount of milk. Marked constipation. Knee joints weak and evidently painful but not swollen. Urine contains a coagulable deposit equal to one third the bulk of entire fluid. Animal killed and autopsied.

The following abnormalities were noted. The rumen contains a very large quantity of hair intimately mixed with the semisolid vegetable contents. The fourth stomach contains a viscid white fluid and some twine from the bedding. Some petechiae in the leafy portion. In the large intestine, feces are dry and formed into hard pellets.

The perirenal fat is edematous, and from ½ to 1 cm. thick, owing to the accumulation of a clear colorless fluid in its interstices. The capsule easily removed. The cortical tissue is very pale, without normal markings and dotted with dark red points, about 1 mm. or less apart. Under capsule are scattering hemorrhages and clots, several in each lobe and from a few to 15 mm. in diameter. On section, the same dark red points throughout cortex. Medulla without noticeable changes. Kidneys, after removal of fat, weigh each 350 grams, *i.e.* they are nearly twice normal weight.

Free hand and frozen sections of these organs show lesions of the epithelium of the convoluted tubules. Within the nuclear membrane is a group of fused disclike bodies of moderate refringence and slightly brownish tint. The individual discs composing the mass are 1 to 2μ in diameter. The masses themselves 5 to 10μ in diameter. Besides these intranuclear bodies there are larger masses of similar material irregular in form gathered in lumina of tubules and blocking them in part or wholly. 5 per cent acetic acid does not disturb them. Normal NaOH acts upon them and after several hours reduces them to heaps of minute granules.

Sections of kidney tissue fixed in Zenker's fluid show a marked irregular dilatation of the convoluted tubules. Nuclear membrane of the epithelial cells irregularly folded, one portion pressed toward the opposite half. They are 2 to 3 times normal size and contain faintly stained foreign masses. The glomeruli are either normal or else contracted and pressed to one side. The glomerular space is filled with a bubbly mass or else with red corpuscles. Hemorrhages are widespread in convoluted tubules, with the glomeruli as the probable source of the blood. The columns of Ferrein appear more compact than is normal, suggesting slight hyperplasia of the stroma. Hyaline casts present. In the collecting tubules hyaline casts numerous.

In certain animals showing renal lesions intranuclear bodies were not detected. However, there were observed casts either in the urine or in the collecting tubules made up of apparently the same material as that within the nuclei of the epithelial cells. Details of these cases are omitted.

An illustration of the association of the lesions of the spotted kidney with the type under discussion is furnished by a calf[8] which received colostrum after a delay of 22 hours. The kidneys of this animal closely resembled those of No. 1511. Intranuclear bodies were present and located chiefly in the labyrinth.

In the course of experiments[9] on the substitution of serum for colostrum a certain number of calves died unexpectedly in the 2nd month of life during the night or quickly after convulsive seizures during the day. In one of the animals thus affected intranuclear bodies were found. The relation between this acute fatal affection and the more slowly developing renal lesions is an open one. There are, however, reasons for tentatively regarding the renal and the convulsive type as allied etiologically. The following case among several is therefore briefly outlined.

Calf 1416.—Holstein heifer, weighing at birth 70–75 lbs. It received in place of colostrum during the 1st day 700 cc. of serum of Cow B (treated with heated *B. coli*). Slight proteinuria on 2nd day disappearing on 3rd day. On the 50th day of life, the calf refuses part of the usual amount of milk and develops symptoms due to irritation of central nervous system. The left ear is held back against head. The calf moves at times in a circle with unsteady gait. The left side of head and trunk is pressed against the sides of stall leading to contusions of head on that side. It is evident after a time that the calf fails to see. The pupils are dilated. Later in the day the animal lies down, with head drawn to the left and right ear twitching. Gets up and walks in a circle. Profuse sweating. The next day calf is unable to swallow its food. Gait unsteady. Forelegs spread apart. Knees and ankles bent. The symptoms remained unchanged until the 4th day. The calf began to take milk from a bottle. Blindness continued. During these days the urine contained about 1 to 1.5 per cent coagulable deposit and the temperature did not rise above 39.5°C. The case was tentatively considered one of botulism poisoning. The specific bacillus could not be demonstrated in the fecal discharges. Constipation was marked and the feces were in the form of small balls.

97th day. Calf has remained blind, but has recovered in other respects. Urine has contained from 1 to 3 per cent coagulable material up to the present. Killed today. The abnormal conditions noticed are as follows:—Digestive tract normal except lower half of large intestine which is filled with very dry fecal balls 1 to 2 cm. in diameter. Slight, rather firm adhesions of right cephalic and ventral lobe of lungs to ribs. Collapse of scattering lobules in cephalic lobe. Both kidneys en-

[8] Smith, T., *J. Exp. Med.*, 1925, **41**, 422.

[9] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, **36**, 453.

larged and with uniformly pale cortex, increased in thickness. Medulla sharply demarcated from it by fullness of vessels. Weight of each kidney, freed from fat envelope, about 305 grams.[10] Some slight adhesions of caudal border of liver to kidneys and other structures. A trace of fat in liver cells. Malpighian bodies of spleen very distinct. Ovaries cystic. Brain and medulla without visible abnormalities.

In free hand and frozen sections of kidneys, the epithelium of the convoluted tubules contains the same kind of material described under Calf 1411. There are no casts of this material in the lumina.

Examination of fixed material from kidneys shows a slight distension of convoluted and straight tubules in limited areas throughout cortex. Scattering interstitial foci of cells made up of polynuclear and some mononuclear leucocytes. The enveloped tubules filled with polymorphs. Epithelium of convoluted tubules with enlarged and irregularly folded nuclei, appearing like collapsed spheres. In certain areas there are intranuclear discs stained reddish. Casts present but in small numbers. Sections of liver, spleen and brain without noticeable abnormalities.

This is the only animal to survive the acute attack. The renal lesions resembled those of the group in which such an attack was not observed. However, in several acutely fatal cases the highly congested kidneys did not show intranuclear bodies.

DISCUSSION

The observations set down in the preceding condensed protocols are published at this time because of the interest manifested in similar structures associated with certain infectious diseases and also because the investigations have been discontinued for the time being. The material is relatively scarce, it has been available at unexpected, inopportune moments, and in certain instances it was undiagnosed during life. It nevertheless contains a certain interest in that it presents several significant problems in renal pathology.

As regards clinical indications it may be said that they were relatively scarce. Proteinuria was present only towards the end of life. The amount of protein was then quite large. Constipation was marked in several animals. The temperature rarely rose more than a degree C. and usually in association with diarrheal conditions.

The intranuclear bodies were observed both in an otherwise still

[10] Normal weight at this age about 200 grams.

normal kidney and in organs markedly altered. The chief characters are enlarged white organs covered with an edematous layer involving the perirenal fat. The cortex is peppered with petechial hemorrhages which on section are seen to be present throughout the cortex. The distension of the entire convoluted tubule system of the labyrinth was present in some, and of only certain portions in others. Isolated collecting tubules were also greatly distended. In the formalin-fixed kidneys, the calices of the pelvis were distended. Inflammatory reactions, such as interstitial focal collections of cells and hyperplasia of stroma, may or may not be present. Hyaline casts are frequently seen, chiefly in Henle's loops. Bacteria are absent both in cultures and in sections of the organ.

The intranuclear bodies are in all cases alike. They are made up of roundish disclike units, 1 or 2μ in diameter, fused into masses containing up to a dozen discs. The fused masses are irregular in form and suggest concretions. Weak acids and alkalies have not dissolved or otherwise changed them materially. They are situated mostly in the epithelium of Henle's loops, more rarely in the convoluted tubule epithelium. These bodies are not of common or frequent occurrence in the calf. The kidneys of many calves killed after 2 months and apparently normal were searched in vain for these bodies. A study of the tables in another publication[11] brings out the fact that the renal lesions were found only in calves in which there was a deviation from the normal colostrum feeding, either by substituting serum, or by delaying the intake of colostrum, or allowing only a small amount of colostrum at the start. However, only a small per cent of these were affected. It is significant that none of the 14 controls[11] allowed to feed normally with the dam for 3 or 4 days were abnormal when kept 4 to 10 weeks.

Thus far no information bearing on the nature of the intranuclear bodies is at hand. Their appearance suggests some form of concretion, deposited or formed within the nuclear membrane. The involvement of the cells may be quite extensive, almost universal, as in No. 1532 (Figs. 1–3).

The writer has been unable to find in medical publications references

[11] Smith, T., and Little, R. B., J. Exp. Med., 1930, **51**, 483.

to renal conditions similar to the ones described, except a paper by Van Leersum.[12] In studying urinary calculi in rats on a vitamin-A-deficient diet he came to the conclusion that the calculi are not merely deposits of urine salts but made up of "epithelial cells impregnated with calcium." He did not, however, trace the process back towards its beginnings in the attached cell.

The effect on the epithelial cells is at first almost negative. Later the cells become hypertrophied as the inclusions grow and project into the lumen of the tubules. In one case a mitotic cell was detected in which the inclusion had been pushed into the cytoplasm. The cell was thus still capable of reproduction. Whatever the final effect, the degenerative process appears to be slow. The end result, as suggested above, is probably the shedding of the cell and its replacement by mitotic multiplication at least for a time.

Taking the various gross and microscopic data into consideration, we may conclude that the main disturbing factor is occlusive and that occlusion is due to a shedding of the involved epithelium with the formation of casts in the tubules and possibly later on concretions obstructing also the ureter. The perirenal edema and multiple petechial hemorrhages favor this theory. The presence of numerous casts in certain cases not here reported made up of disclike bodies is contributory evidence. It is conceivable that in the animal (No. 1532) in which the epithelium of the still normal kidneys was universally involved, the simultaneous discharge of these concretions might cause blocking of the entire excretory system. Complications of this type of injury, with lesions due to the "spotted kidney" form of infection with *B. coli*, are evident in some of the protocols.

In accordance with prevailing data and concepts, the interpretation of the intranuclear inclusions as the result of an infectious process is tempting. This theory is favored by the fact that in general the deprivation of colostrum leads to a variety of infectious diseases, and that any evidence that this deprivation causes a recognizable deficiency disease is thus far lacking. If the process is infectious it could best be assumed as due to a filtrable virus occupying the nucleus and causing the changes leading to the concretions. Moreover, like the other infec-

[12] Van Leersum, E. C., *J. Biol. Chem.*, 1928, **79**, 451.

tious diseases brought to the surface by the colostrum experiments, it would have to be regarded as of low virulence, appearing only sporadically under the usual environmental conditions and perhaps very rarely in older animals. Since the subject of spontaneous renal diseases of lower animals is still in the descriptive stage, we must await further developments and the increased prevalence of this type of disease which will permit a wider, more detailed study.

Another quite different theory to account for the renal lesions is the early absorption of *B. coli* toxins from the digestive tract. Most of the animals whose protocols are given went through an early attack of liquid stools. Those that die in this stage (scours) have intensely congested kidneys. The effect of the *B. coli* toxins eliminated through the kidneys may in certain surviving cases bring about the peculiar epithelial abnormalities described. To prove or disprove this hypothesis, any more definite experimentation than the one followed (withholding colostrum, etc.) cannot be carried out because the calf after the first week cannot be infected with *B. coli* by feeding. The intravenous injection of filtrates is not a complete substitute since the toxins formed in the ileum may be either more potent or different from these in the culture tube.

Intermingled with cases of the renal disease were those in which convulsive seizures coming on suddenly and accompanied by various forced movements indicating irritation of the central nervous system were the chief symptoms. These are twitching of one ear or tail, movements of the legs, staggering gait, difficult swallowing, disturbance of vision, grating of teeth, convulsion and death within one half to several hours after the sudden beginning of the attack. The postmortem changes are hemorrhages in the intrathoracic portion of thymus, petechiae on the epicardium and large vessels, and pulmonary congestion. In 6 animals thus affected, only one (No. 1416) did not die within a few hours of the beginning of the attack. This animal became blind and lingered along for 47 days after the attack when it was killed. Various causes suggested themselves, among them botulism, status lymphaticus, acute poisoning, and anaphylactic reactions. In two of these cases intranuclear bodies were present. The rest had not been examined as thoroughly for such bodies, since the earliest cases were regarded as due to poison. Tentatively the hypothesis is put forth

that this group of symptoms may be the earlier acute fatal stage of the renal disease and that the inclusion bodies develop in the less acute type. Or, a similar intranuclear affection of epithelial cells elsewhere in the body thus far overlooked, may lead to the convulsive type.

CONCLUSION

Renal lesions chiefly the result of obstructive processes were associated with intranuclear bodies in the epithelium of straight and convoluted tubules. In one animal these bodies were found in large numbers in otherwise still normal organs. The affected animals had been fed cow serum in place of colostrum.

EXPLANATION OF PLATES

PLATE 9

FIG. 1. Frozen section of fresh kidney of Calf 1532 in physiological salt solution, showing the almost universal presence of intranuclear bodies in the straight tubules. × 205.

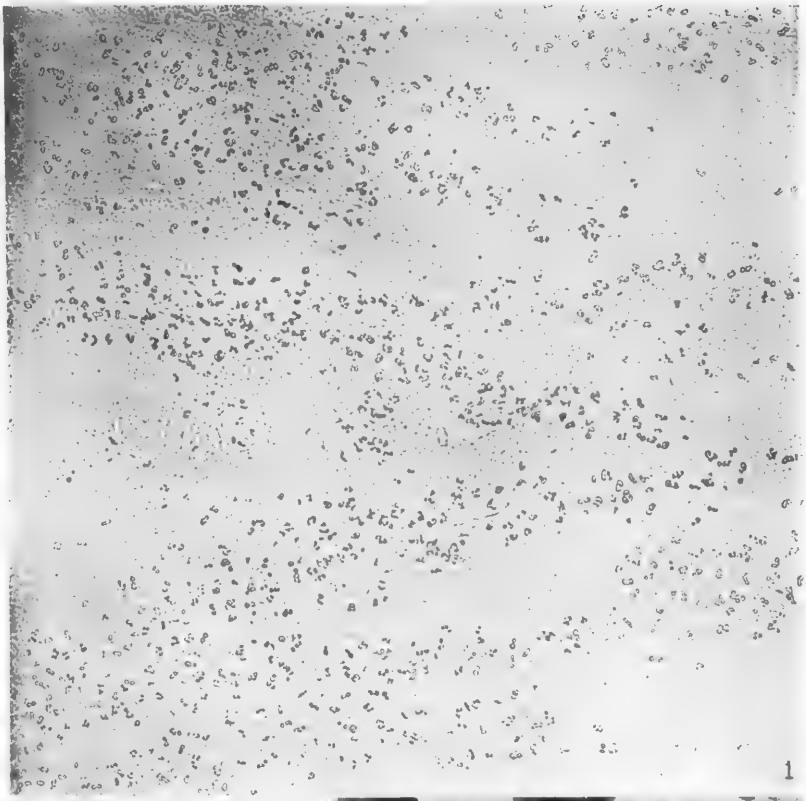
FIG. 2. Film prepared by scraping the cut surface of the fresh kidney, drying the film, passing it through absolute alcohol and water, staining in hematoxylin and eosin, clearing, and mounting in balsam. × 560.

FIG. 3. The same preparation. × 1920.

PLATE 10

FIG. 4. Frozen section of formalinized kidney of Calf 1511. Mounted in water. × 200.

FIG. 5. Frozen section from the same kidney, showing the intranuclear site of the inclusion bodies. × 350.



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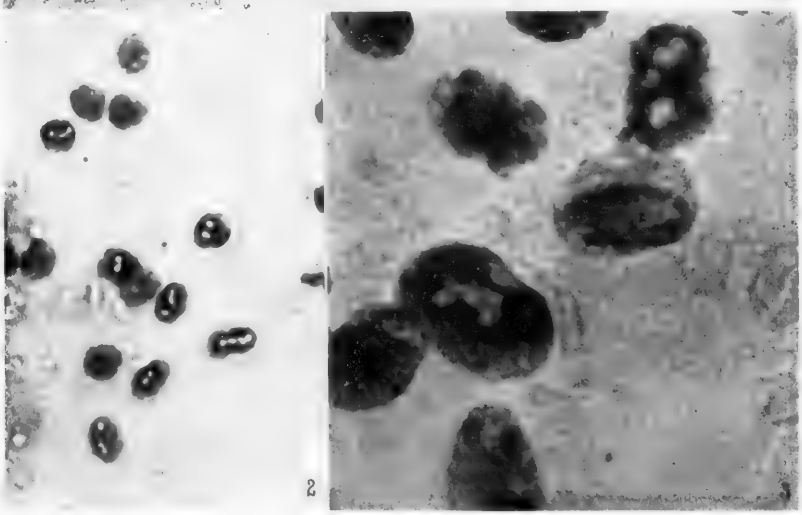

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(Smith: Immunological significance of colostrum. III)

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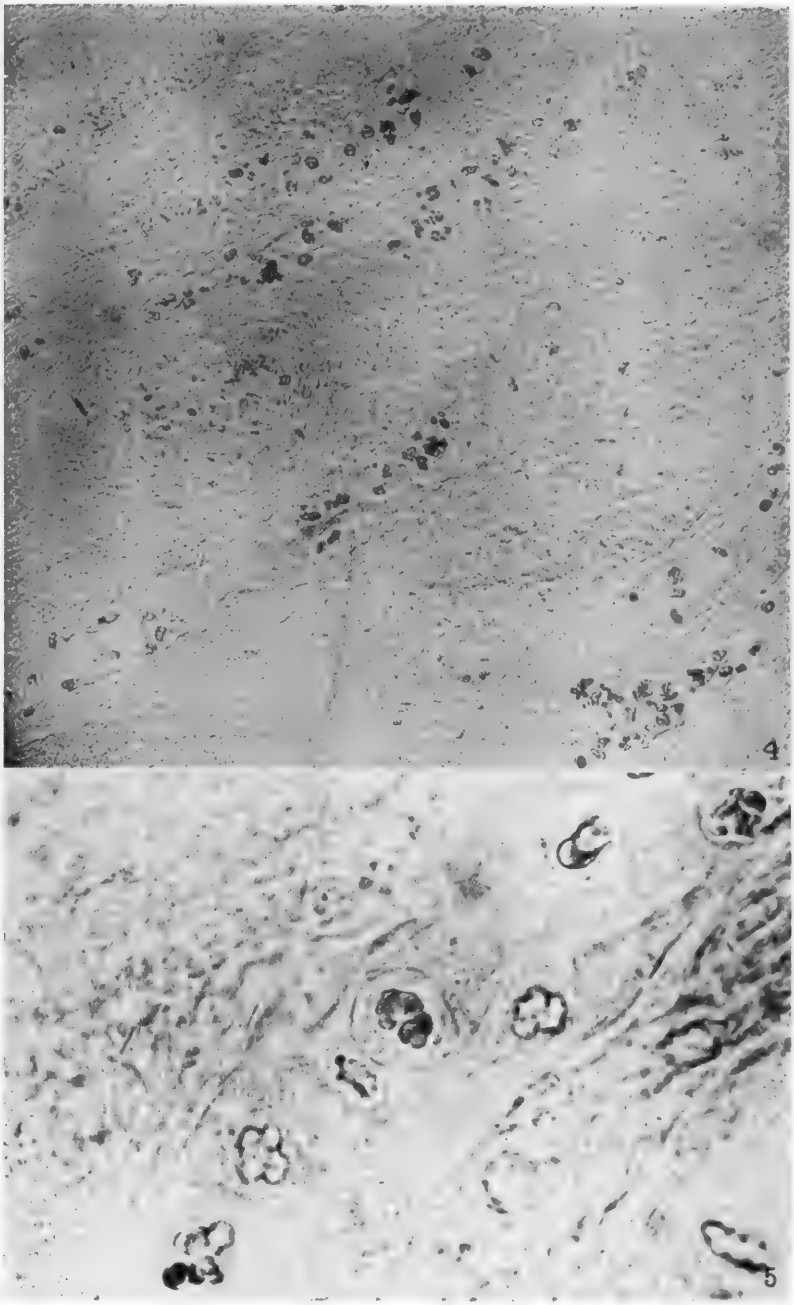

(Smith: Immunological significance of colostrum. III)

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STUDIES ON VITAL STAINING

IV. India Ink and Brilliant Vital Red. Importance of Considering Liver Excretion in the Study of "Blockade of the Reticulo-Endothelial System"

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INTRODUCTION

In previous papers of this series we have presented experiments dealing with the distribution in the body of certain dyestuffs when given intravenously (1). The two dyes, brilliant vital red and Niagara sky blue, like many other acid dyestuffs are gradually taken up by phagocytic cells in various parts of the body ("reticulo-endothelial system" of Aschoff). They differ from many dyes of this group in being taken up rather slowly by these cells, so that the concentration of dye in the plasma may be followed over a period of many hours before the concentration becomes too low to be determined by colorimetric methods. At first the tissues remove either dye quite rapidly from the blood stream, but later on as the tissues become more deeply stained they become less active, and at such concentrations of dye in plasma they take up dye less readily than they normally would. This sluggishness or "inhibition" of tissue activity represents in reality an equilibrium in dye concentration between tissues and body fluids, for if more of the dye be injected the tissues will take up much of it. The dye continues to pass into the tissues until a second equilibrium point is reached at a higher level.

These two dyes are rather similar in their general physico-chemical properties and we need not be surprised that they are deposited within the same identical phagocytes (2). Our evidence shows (2) that the presence of one dye within these cells does not prevent the cells from taking up the other at the normal rate. Furthermore, the phagocytes can take up both dyes simultaneously when they are injected together. The cells respond without hesitation to this double task, with the result that each dye is removed from the plasma with normal speed, not only at first when the cells are free of dye, but later on as well, when the cells contain considerable amounts of each dye. The two dyes within the cell do not appear to exert any mutual antagonism which would slow up the rate of storing dye or hasten the onset of the equilibrium for either dye.

It is common knowledge that enormous numbers of phagocytes exist within the connective tissues and within the lymph nodes. These cells do not lie in intimate contact with the blood stream as do the Kupffer cells of the liver or certain phagocytic cells of spleen or bone marrow. It has never been clear whether or not cells of this latter type are really more actively phagocytic than the cells more distantly removed from the circulating blood. The fact that they take up foreign materials so promptly may merely be a correlary of their accessibility to the blood stream and hence to the source of the foreign materials. At a later date we shall present data to show that after removal of liver or spleen, brilliant vital red is still quite rapidly removed from the circulating blood. The removal of the liver with all of its Kupffer cells or the spleen with its numerous phagocytes does not appear to cause great impairment of the phagocytic system as a whole. We may concede that as individuals the phagocytes of liver or spleen are perhaps more highly endowed types of phagocytes, still it is our belief that the phagocytes of the connective tissues generally are so extremely numerous that much and probably most of the total phagocytic power in the body resides in them.

When India ink is injected into the blood stream the particles of carbon tend to agglutinate, and very few of them succeed in passing through the capillary walls. They are promptly taken up and stored in those phagocytes which lie in intimate contact with the blood stream. Such injections offer us an opportunity to test our ability to "block" these particular cells without in any way involving the phagocytes which lie well outside the vessel walls. To test the phagocytic response of these ink-laden cells one should inject some second foreign substance which would be taken up by these cells, and by them alone. Unfortunately most if not all substances of this class are in such an unstable state of dispersion that they precipitate out in the blood stream. Many of these particles are caught and held mechanically in the capillaries, so that speed of removal cannot be used as a measure of phagocytic activity. On the other hand most dyestuffs, like brilliant vital red, reach vast numbers of phagocytes besides those which contain ink. Our experiments with liver removal and with splenectomy have lead us to believe that these extravascular cells possess a large total phagocytic power. We could hardly expect the presence of ink in the smaller group of cells to reduce the total bodily response very markedly, even though the ink did block the cells which take it up. For these reasons we were not prepared to find that India ink does actually delay the rate at which brilliant

vital red leaves the circulating blood. At first we seemed compelled to attribute this to defective phagocytosis, but later on we found that the ink injection inhibits the excretion of dye into the bile. The longer retention of dye in the plasma can be explained by defective elimination on the part of the liver.

From these observations it became evident that peculiarities of liver excretion demand careful attention. It is necessary to have accurate data on this subject if we are to interpret any data on the rate at which dye leaves the blood stream. Obviously, changes in this rate cannot be ascribed to changes in phagocytic response unless we can show that liver excretion is not responsible. Much of the literature of "blockade" does not take these considerations into account. It is not sufficient to assume that liver excretion is constant or that it is proportional to dosage of the dye. In fact this latter supposition is definitely contrary to fact (3). With small doses as much as 30 per cent may be eliminated into the bile during the first day, whereas with larger doses the percentage output may not be more than 15. After chronic vital staining or after single large injections the dye may be less efficiently removed from the blood stream, but as in the case of India ink injections, this must be attributed to the inefficient liver excretion and not to "blockade" of phagocytes.

Methods

Details regarding care of the dogs and the collection and analysis of blood samples have been given elsewhere (1).

The India ink used was Higgins' American India ink. The exact composition of this ink has never been published. It contains about 10 per cent of solids. We have found that the carbon can be precipitated with dilute acids. If this black mass be resuspended in water and again precipitated with dilute acid and then washed with alcohol and dried it will be found to represent about 7 per cent of the original ink. This figure may be a trifle too high but we feel that most of the impurities have been separated from the carbon by this process of precipitation and washing. Kjeldahl determinations show that the precipitated mass contains almost no nitrogen.

The ink was injected either without dilution, or in some cases after dilution with an equal volume of normal saline. Since much of the ink is precipitated in the blood stream and is promptly filtered out by the capillaries we have not been interested in following the

rate at which the ink leaves the blood stream, for such studies give no information regarding the rate of true phagocytosis by the tissues. We may note in passing that in our experiments the blood stream was regularly free of carbon particles within an hour or less. We have taken advantage of this fact and have timed the administration of ink in such a way that ink would never be present in the plasma to interfere with the regular colorimetric determinations of dye.

Bile was collected for study in certain of the dogs.

A slight modification of the bile fistula operation of Rous and McMaster (4) has been made by Smith, Groth and Whipple (5) and their technic was used in the present experiments. By this method bile is collected by a cannula inserted into the common bile duct and is brought to the outside and collected in a rubber bag from which it can be removed daily, or oftener if desired. The rubber bag is protected from injury by suitable dressings which are placed about the dog. Bile can be collected whenever desired over a period of many weeks. The dog remains in good condition throughout unless pathogenic organisms gain entrance from the outside to the bile passages, but under favorable conditions, using aseptic technique in making the bile collections, the bile may be kept sterile for a number of weeks.

The quantitative analysis of brilliant vital red present in the bile collected offers certain difficulties. We had hoped that it would be possible to make estimations by means of the spectrophotometer, using a previously described method (6) for the quantitative analysis of colored mixtures. To investigate this possibility artificial mixtures of bile and brilliant vital red were made, but it was found that the absorption curves of the two substances were not strictly additive, though approximately so in certain portions of the spectrum. There was the further complication that the absorption bands of these two substances overlap to a very considerable extent. Both absorb light in the green and blue much more than in the red. Although the absorption curves for the two substances are by no means identical, yet the relations are such that no great precision can be expected by spectrophotometric methods. For these reasons we have adopted the well known and simple method of analysis based on the use of the "color comparator."

Two series of standard solutions are prepared, one containing bile in varying concentration from 1 to 10 to 1 to 60, the other containing brilliant vital red vary-

ing from 1 to 15 mg. per liter. The unknown is diluted to a varying degree but usually to about 1 to 30 and is poured into a test tube and placed in a rack behind another tube containing water. The light transmitted through these two tubes is then matched in quality and in intensity with that produced by placing in allignment two of the standard dye and bile tubes. By making successive trials a combination of a dye and a bile tube can be found which matches very closely the unknown, both as regards shade and intensity. Obviously, care must be taken that all of the tubes used are of the same diameter. The method is quick and simple and when dye is present in the bile in considerable quantities—200 mg. per liter or more—the error is somewhat less than 10 per cent, as can be shown by test analyses of known mixtures of dye and bile. The physiological changes which we will discuss are several times this experimental error, so that the method of analysis answers fully the present requirements.

EXPERIMENTAL

Brilliant vital red injected into a dog by the intravenous route disappears from the circulation at a rate which varies slightly with different individuals, but we have numerous experiments to show that for a given dog the experiment may be repeated at intervals of weeks or months with almost identical results. The elimination rate for the dog may be determined by a preliminary test dose of dye, and after several weeks the tissues will be almost entirely free of the latter and we are at liberty to repeat the experiment either with or without the introduction of procedures calculated to influence this established disappearance rate of dye.

Dog 24-74. Shepherd. Weight 32 kg. (See Table 41.)

February 3, 1928. Control experiment to determine normal elimination from the blood stream of brilliant vital red. Twenty cubic centimeters of an aqueous 2 per cent solution of brilliant vital red were injected into the jugular vein. At varying intervals thereafter samples of blood from the opposite jugular vein were taken into oxalate solution, and after centrifugalization the concentration of dye in the supernatant plasma was determined by means of the spectrophotometer. The results are given in the second column of Table 41.

March 25, 1928. Experiment to determine the effect of ink injection on the disappearance rate of dye. At 9:52 A.M. 20 cubic centimeters of 2 per cent brilliant vital red were injected. The 5 minute sample was taken at 9:57 A.M. and immediately following this, 10 cc. of Higgins' American India ink were injected into the jugular vein. The ink disappeared rather rapidly from circulation and the blood samples taken 1 hour later and subsequently contained no ink whatever. The amount of dye in the various samples of plasma taken during this

elimination period of 4 days is shown in the third column of Table 41. At no time during or following the injection of the dye or ink was there any evidence of sickness on the part of the dog.

May 20, 1928. Dye injection to test elimination rate 2 and 6½ months after injection of India ink. The experiment was carried out as on February 3. The results are shown in the fourth and fifth columns of Table 41.

TABLE 41

Dog 24-74. Effect of Ink on Disappearance of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma			
	Dye without ink	Dye with ink*	Dye 2 months after ink	Dye 6½ months after ink
	mg.	*mg.*	*mg.*	*mg.*
5 min.	260	260	240	255
1 hr.	225	225	225	215
6 hrs.	145	160	160	155
24 hrs.	37	114	72	43
48 hrs.	18	55	26	17
72 hrs.	8	32	16	13
96 hrs.	4	11	5	3

* Ink 5 mins. after dye injection.

TABLE 42

Dog 24-96. Effect of Ink on Disappearance of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 4½ months after ink
	mg.	*mg.*	*mg.*
5 mins.	480	460	460
1 hr.	335	460	390
6 hrs.	310	350	315
24 hrs.	160	240	180
48 hrs.	74	140	98
72 hrs.	57	91	54
96 hrs.	31	81	48

* Ink 5 mins. after dye injection.

Dog 24-96. Shepherd. Weight 15 kg. (See Table 42.)

February 17, 1928. Control to determine rate at which brilliant vital red normally leaves the blood stream. Twenty cubic centimeters of 2 per cent dye injected into jugular vein. Samples of blood taken at intervals during the next 4 days

were received into isotonic sodium oxalate (1.6 per cent) solution. Each sample was promptly centrifuged. The amount of dye in the supernatant plasma in each case is shown in the second column of Table 42.

May 27, 1928. The effect of an injection of India ink upon the disappearance rate of the dye. As in the case of dog 24-74, 20 cc. of brilliant vital red were injected and 5 minutes were allowed for admixture with the circulating blood. Immediately after the 5 minute sample of blood had been taken for dye analysis, an injection of 10 cc. Higgins' American India ink was made. That the ink particles were rapidly swept out of the blood stream is shown by the fact that a blood sample taken an hour later contained no trace of carbon. The rate of dye elimination from the blood is indicated by the figures shown in column 3 of Table 42. There were no signs that either dye or ink were toxic to the animal.

TABLE 43

Dog 25-29. Effect of Ink on Disappearance of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 4½ months after ink
	mg.	*mg.*	*mg.*
5 mins.	370	385	320
1 hr.	325	350	275
6 hrs.	205	320	215
24 hrs.	102	150	109
48 hrs.	50	94	61
72 hrs.	33	65	37
96 hrs.	17	48	20

* Ink 6 hours before dye injection.

October 7, 1928. Disappearance rate of dye 4½ months after injection of India ink. The experiment is identical to that carried out on February 17. The concentration of dye in the plasma in each of the samples taken is shown in column 4 of Table 42.

Dog 25-29. Collie. Weight 28 kg. (See Table 43.)

March 2, 1928. Control to determine the rate at which brilliant vital red normally leaves the blood stream. Twenty-four cubic centimeters of 2 per cent brilliant vital red injected into the jugular vein. In the second column of Table 43 is shown the concentration of dye in each of a number of samples of plasma collected during the next 4 days.

May 30, 1928. Effect of an injection of India ink upon the disappearance rate of the dye. Unlike the experiments on dogs 24-96 and 24-74 the regular dose of 10 cc. Higgins' American India ink was given 6 hours *before* making the dye

injection. The latter consisted of 24 cc. of 2 per cent brilliant vital red, also given intravenously. No evidence of toxicity either on the part of ink or dye. Plasma collected at varying intervals following the injection of dye was analyzed for its dye content. The results are shown in the third column of Table 43.

October 6, 1928. Disappearance rate of dye 4½ months after injection of India ink. The experiment is similar to that performed on this dog on March 2. The concentration of dye in each plasma sample collected is shown in the fourth column of Table 43.

Dog 27-243. Male collie. Weight 24 kg. (See Table 44.)

April 29, 1928. Control to determine the rate at which brilliant vital red normally leaves the blood stream. Twenty cubic centimeters of 2 per cent aqueous brilliant

TABLE 44

Dog 27-243. Effect of Ink on Disappearance of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 4 months after ink
	mg.	*mg.*	*mg.*
5 mins.	250	300	335
1 hr.	225	280	295
6 hrs.	155	205	185
24 hrs.	70	105	76
48 hrs.	30	74	26
72 hrs.	22	44	17
96 hrs.	11	37	6

* Ink 24 hours before dye injection.

vital red injected intravenously. In column 2 of Table 44 are given data showing the concentration of the dye in the plasma in various samples subsequently taken.

May 30, 1928. Effect of an injection of India ink upon the disappearance rate of the dye. Unlike the other cases the 10 cc. dose of American India ink was given intravenously *24 hours before* the dye was given. As in the control experiment one month previously, 20 cc. of the 2 per cent aqueous brilliant vital red were given. The plasma collected at varying intervals following the injection of dye was analyzed for its dye content. The results are shown in column 3 of Table 44. There was a short period of deep breathing and some nystagmus following the injection of ink. Prompt and complete recovery.

October 7, 1928. Disappearance rate of dye 4 months after injection of India ink. The experiment is identical to the control carried out on April 29. The results are shown in the fourth column of Table 44.

We have a number of experiments, all of which go to show that the intravenous injection of India ink slows up very markedly the rate at which brilliant vital red leaves the blood stream. We submit four such experiments (Tables 41–44 inclusive). The results of these experiments are in agreement and the outstanding features are averaged and shown graphically in Chart 4A. It will be observed that the initial blood sample taken 5 minutes after the injection of dye shows the plasma to contain nearly 350 mg. dye per liter plasma. In all cases the greater part of the dye leaves the blood stream during

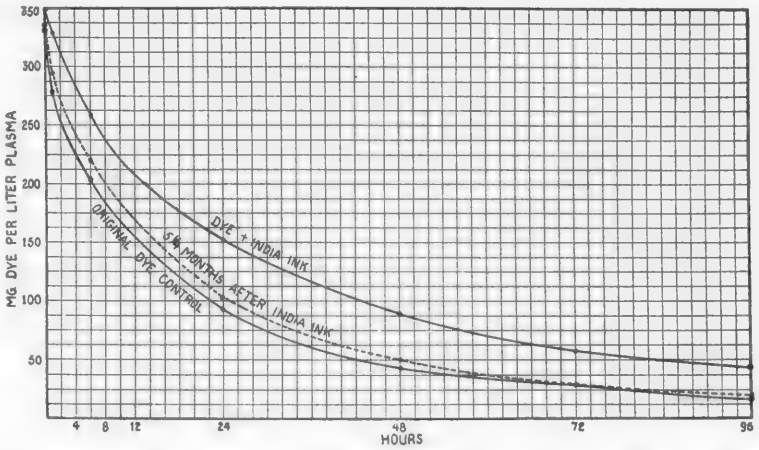

CHART 4A. Elimination of brilliant vital red from plasma. Effect of India ink. (Curves represent the averages of four dogs—Tables 41–44 included.)

the course of the next four days, but there is a most striking retardation in the elimination rate when a small dose of ink is injected at the beginning of the experiment. This retardation begins almost at once and at the end of 48 hours there is twice as much dye in circulation as in the normal control determinations, and later on the discrepancy is even somewhat greater. The change is several times what we know to be the maximum experimental error.

Large doses of India ink are somewhat toxic but the small doses used by us did not disturb the animal. In all cases they ran about in a normal manner and ate the regular ration of hospital scraps.

We have noted occasionally that there is some retardation in coagulation of blood following injection of ink, but hemorrhages into skin, mucous membranes or intestinal tract were never noted. We have abundant proof that the elevation of the dye elimination curve following ink is not due to a decrease in the blood volume, for the centrifuged samples of blood showed a constant ratio of cells to plasma throughout the experiment. Furthermore in two of the experiments, (Tables 43 and 44), the ink was given a number of hours before the dye was injected, and in neither case was the initial dye reading greatly elevated as one would expect if the ink had caused a reduction

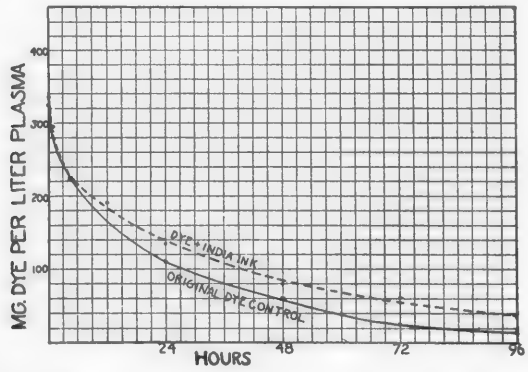

CHART 4B. Dog 27-229. Elimination of brilliant vital red from plasma. Normal control contrasted to curve showing ink given six hours after dye.

in blood volume. There can be no doubt whatever that the small dose of ink has brought about a true retention of dye in the blood stream. Another experiment showing this phenomenon is shown in Chart 4B. Here the administration of ink was delayed until 6 hours after the dye had been introduced into the blood stream. We note that during these first 6 hours the dye curve falls off at a rate which is almost identical to the control done 5 weeks previously, but from the moment the ink was injected we note that the two curves separate, indicating that the ink has acted very promptly in slowing up the disappearance rate of the dye.

Dog 27-229. Weight 22.5 kg. (See Chart 4B.)

April 29, 1928. Control to determine the rate at which brilliant vital red normally leaves the blood stream. Twenty cubic centimeters of 2 per cent dye were injected intravenously. There were no untoward symptoms following this procedure. The concentration of the dye in the plasma at different intervals is recorded in Chart 4B.

June 2, 1928. Effect of an injection of India ink upon the disappearance rate of the dye. Twenty cubic centimeters of 2 per cent dye were injected intravenously, but *6 hours after the dye*, 10 cc. of American India ink were given. During the ten minutes following the ink the animal was dyspneic, his muscle tone diminished and for the rest of the day he seemed somewhat depressed. However, the next day he appeared quite well.

The concentration of the dye in the circulation at different intervals is shown by the dotted line in Chart 4B.

We call especial attention to the results shown in Table 44. In this case the ink was injected 24 hours before the dye was given. The ink caused only slight and very transient clinical disturbance. Of particular significance is the fact that we note well marked delay in the rate at which the dye leaves the blood stream. In fact the delay seems to be as great as when ink and dye are given on the same day. It is clear that the action of ink in retarding dye elimination persists for several days at least. When a test dose of dye was injected 5 months after ink injection (Chart 4A) the rate of dye elimination appears to be almost if not entirely normal. In fact we believe that recovery is practically complete in 3 weeks (Chart 4C) although the departure from normal at that time seems to be slightly more than we can charge to experimental error. We have other experiments which show retardation 3 weeks after ink even more distinctly than this; still we feel that the effect of the ink largely wears off after the first week.

Dog 28-165. Mongrel. Weight 15.5 kg. (See Table 45.)

May 11, 1929. Control to determine rate at which brilliant vital red normally leaves the blood stream. Fifteen cubic centimeters of 2 per cent dye injected into jugular vein. At intervals during the next 4 days samples of blood were drawn into isotonic sodium oxalate and after centrifugalization the amount of dye in the plasma was determined. The results are shown in the second column of Table 45.

June 1, 1929. The effect of an injection of India ink upon the disappearance rate of the dye. As on May 11, 15 cc. of 2 per cent brilliant vital red were injected

into the jugular vein. Five minutes were allowed for complete admixture of the dye with the circulating blood and at the end of this time a sample was taken for analysis. Immediately thereafter 8 cc. of Higgins' American India ink were injected. Dog was very slightly depressed for about an hour only. No vomiting.

TABLE 45

Dog 28-165. Effect of Ink on Elimination of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 3 weeks after ink
	mg.	*mg.*	*mg.*
5 mins.	520	455	495
1 hr.	430	390	395
6 hrs.	245	300	305
24 hrs.	160	165	160
48 hrs.	69	110	68
72 hrs.	43	69	43
96 hrs.	28	43	30

* Ink 5 mins. after dye injection.

TABLE 46

Dog 28-184. Effect of Ink on Elimination of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 3 weeks after ink
	mg.	*mg.*	*mg.*
5 mins.	410	380	375
1 hr.	340	370	325
6 hrs.	210†	275	230
24 hrs.	96	165	109
48 hrs.	45	86	49
72 hrs.	39	45	24
96 hrs.	39	28	16

* Ink 5 mins. after dye injection.

† By interpolation.

Samples of blood taken an hour after these injections as well as those taken later contained no dye in the plasma though smears showed some carbon in the white cells. The dye concentration was determined at intervals during the following 4-day interval. The results are shown in the third column of Table 45.

June 22, 1929. Disappearance rate of dye 3 weeks after injection of India ink.

The experiment is identical in technical details to that carried out on May 11. The concentration of dye in the samples taken for analysis is shown in the fourth column of Table 45.

Dog 28-184. Mongrel, male. Weight 16.5 kg. (See Table 46.)

As regards dates, dosage and the taking of samples this experiment is in every way identical to that on dog 28-165. The details are given in the protocol of that experiment. As in that experiment there was slight transitory clinical disturbance following the injection, but later on in the day the dog ate well and was normally active. The dye readings in the plasma are given in Table 46.

The fact that an injection of India ink will retard the rate at which brilliant vital red leaves the blood stream might be thought to prove

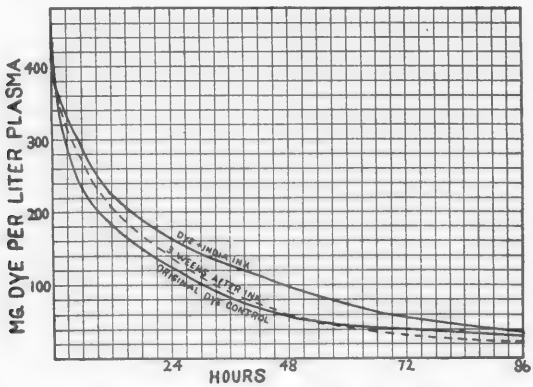

CHART 4C. Elimination of brilliant vital red from plasma. Effect of India ink. Almost complete recovery shown within three weeks. (Curves represent the averages of two dogs—Tables 45 and 46.)

that the ink-laden phagocytes were partially blocked against the entrance of the dyestuff. Indeed, this was our original view. The evidence seems more convincing than much of that commonly offered to support the blockade theory. Our doubts were aroused in the course of studies which we were carrying out on bile fistula dogs. In the course of these experiments it was found that India ink possesses a remarkable ability to slow up or prevent the passage of the dye into the bile. In some cases this effect was associated with considerable diminution in the total volume of bile output per day,

but in many cases the normal amount of bile was excreted. Ordinarily 5 or 10 cc. of ink were injected, and as a rule about twice this amount of two per cent brilliant vital red. In all cases it was clearly seen that for 24 or 48 hours the bile recovered from the animal was of the normal greenish yellow color, quite in contrast to the orange or cherry red colored bile obtained when dye alone was injected. Obviously, such dye retention within the body may well account for the longer retention of dye in the plasma.

In Tables 47 and 48 we present the results of two experiments of the sort just described. In the first, only 100 mg. of dye were injected but we noted that in the normal control period the bile was brightly colored for some days. More dye was eliminated in the first 24 hours than later on, but considerable amounts are present for some time. In all, about half of the dye was recovered in the course of the first 6 days. In the second half of the experiment ink was given along with the dye, and here we noted almost complete failure of dye excretion during the first 24 hours; nor is the inhibition entirely transitory, for the total excreted during the first 4 days is less than half the amount normally excreted in the first 24 hours. Despite this remarkable inhibition in dye excretion we observed no evidence of toxicity on the part of the ink or dye; in fact the dog ate normally throughout the experimental period and the normal amount of bile was excreted. We have analyses to show that in such cases the total output of bile pigments and bile salts also approximates the normal, though the results are somewhat variable. These facts suggest that there is no overwhelming injury to the liver. It would appear that there is a dissociation of functions, for ink seems to inhibit dye excretion without necessarily affecting certain other well established functions. Experiment 32 (Table 48) differs from this experiment in minor respects only. The amount of dye given was three times as great, and as a result we note rather large amounts of dye excreted in the control period. Again, the effect of ink is to suppress the elimination of dye into the bile. The suppression is practically complete for 48 hours, and along with this we note that the daily volume of bile is markedly reduced. Later on the volume of bile returns to normal or slightly above normal, and we observe that some dye is being eliminated, though this activity is rather transitory. In this

case nearly 85 per cent was eliminated during the 9 day control period, but influenced by ink, the liver secreted only one-fourth as much in a similar period.

The dye thus retained within the body becomes distributed between plasma and tissues, coloring each more intensely than normal. We see in these observations a ready explanation for the fact that following an ink injection the dye leaves the blood stream much more slowly than normal, and we feel it unnecessary to suppose that ink has impaired the capacity of the phagocytic system for storing dye.

TABLE 47

Experiment 31. Effect of 5 Cc. India Ink on Excretion of Brilliant Vital Red

	Volume of bile eliminated	Dye per 1000 cc. bile	Total 24 hour dye output
	cc.	*mg.*	*mg.*
Dye (100 mg.) injected without ink			
1st day	85	240	20
2nd day	75	140	11
3rd day	80	120	10
4th day	75	40	3
5th day	60	30	2
6th day	80	15	1
Dye (100 mg.) injected along with ink (5 cc.)			
1st day	85	0	0
2nd day	85	40	3
3rd day	85	40	3
4th day	75	40	3

Dog is a 13 kg. female terrier.

We have been concerned with the question of how it is that ink so completely prevents the excretion of a dye by the liver. There is little to indicate that ink is a liver poison, for we often see apparently normal elimination of bile pigments and bile salts despite the inability of the liver to excrete dye for several days, and with these small doses of ink the dog gives little or no external evidence that the ink is toxic. Work still in progress seems to negate our earlier supposition that the particles of carbon are responsible. We hope to report our

findings more fully in the future, but the evidence seems to indicate that other substances within the ink may be to blame. Finely ground graphite suspended in acacia may be injected without impairing the ability of the liver to excrete dye. Unless such mixtures are made rather strongly alkaline they are quite unstable and the particles are

TABLE 48

Experiment 32. Effect of 7.5 Cc. India Ink on Excretion of Brilliant Vital Red

	Volume of bile eliminated	Dye per 1000 cc. bile	Total 24 hour dye output
	cc.	*mg.*	*mg.*
Dye (300 mg.) injected without ink			
1st day	100	400	40
2nd day	120	400	48
3rd day	110	360	40
4th day	125	240	30
5th day	110	240	26
6th day	130	200	26
7th day	115	160	18
8th day	130	120	16
9th day	120	80	10
Dye (300 mg.) injected along with ink (7.5 cc.)			
1st day	31	0	0
2nd day	23	0	0
3rd day	56	20	1
4th day	140	40	6
5th day	140	120	17
6th day	140	80	11
7th day	140	80	11
8th day	140	80	11
9th day	130	40	6

Dog is a 13 kg. female terrier.

rather large, but we have observed that the particles are distributed about the body in the same manner observed after India ink injections and in each case the Kupffer cells of the liver are heavily laden with carbon particles. These experiments suggest strongly that the inhibitory effects of India ink must be sought in elements other than in the carbon.

This conclusion is reinforced by several experiments in which we have precipitated the carbon from India ink and have observed that the brownish colored fluid so obtained was effective in inhibiting dye excretion by the liver. The carbon of the ink was precipitated by the addition of a known amount of dilute hydrochloric acid and after centrifugalization the supernatant fluid was kept and later combined with certain brownish soluble ingredients which can be extracted from the mass of carbon with the aid of alcohol and of dilute acid. The alcohol was evaporated off and the various carbon-free fractions combined, and enough standard sodium hydroxide added to restore the original alkalinity. As in the case of India ink this solution may be given intravenously without producing any external manifestations of toxicity, but dye elimination by the liver is almost as completely inhibited as in the case of the original untreated ink. It is well known that India ink is rather strongly alkaline. Titration shows that it is neutralized by an approximately equal volume of N/14 hydrochloric acid. We have injected 10 cc. of N/14 sodium hydroxide into the blood stream without observing any decrease in the rate at which the liver will excrete brilliant vital red, simultaneously injected. We do not believe that the alkalinity of the ink is responsible for the inhibition observed with ink injections.

SUMMARY

When brilliant vital red is injected into the blood stream of dogs much of it is slowly taken up into numerous phagocytes scattered throughout the tissues ("reticulo-endothelial system" of Aschoff).

The rate at which the dye leaves the blood stream is determined in large part by the action of these phagocytic cells, but the excretion of dye into the bile is also in part responsible for the loss of dye from the plasma.

The injection of a small amount of India ink into the blood stream results in lowering the rate at which the dye disappears from circulation. The fact that much of the carbon of the ink is promptly taken up by the phagocytes would lead one to suspect that they were saturated with foreign materials, or "blocked" against the entrance of dye, but it is shown that the ink causes a remarkable inhibition of the excretion of dye into the bile, and this alone seems to account for

the longer retention of dye in the blood stream. There is no evidence that any of the retention is due to defective activity on the part of the phagocytes.

Thus, prolonged retention of foreign materials in the blood stream cannot be cited to prove "blockade of the reticulo-endothelial system" unless one can rule out such peculiar reactions on the part of excretory organs. It is felt that the literature of "blockade" should be studied with such sources of error in mind.

Preliminary studies indicate that the suppression of dye excretion by the liver is not due to the carbon content of the ink. Studies of other components of the ink are now in progress.

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STUDIES ON VITAL STAINING

V. Double Staining with Brilliant Vital Red and Niagara Sky Blue. Correlation of Histological with Physiological Data

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INTRODUCTION

After repeated daily intravenous injections of brilliant vital red, the skin, the connective tissues and various internal organs of dogs take on a deep red coloration. This color is due in small measure to the diffuse staining of certain noncellular elements, particularly of the elastic fibers, but in greater part to the deposition within certain cells of great numbers of fine granules of the dye. The staining of elastic fibers, though slight, is well seen by gross inspection of the walls of the larger arteries. Microscopic examination shows quite clearly that the elastic fibers so abundantly present are diffusely and uniformly tinged with dye, there being no evidence that the dye is deposited in granular form within these fibers. Bouffard (1), Goldmann (2), Schulemann (3), Pappenheim and Nakano (4) and many others have observed the diffuse staining of elastic fibers with trypan blue, though pyrrol blue gives no such staining (4), nor does lithium carmine (5) though both of these dyes are excellent "vital stains." Occasionally one can see that brilliant vital red has tinged the delicate ramifying elastic fibers present in the areolar connective tissues but a glance through the microscope shows that the dye, like the other dyes just mentioned, is stored largely in granular form in the phagocytic cells to which we have referred. This segregation of dye within living cells has been the subject of innumerable investigations. For many of the details we refer to the reviews by Möllendorff (6), Aschoff (7), and Börner-Patzelt, Gödel, and Standenath (8).

Amongst the most active of these phagocytic cells are the Kupffer cells of the liver. Unlike many of the cells of connective tissues they lie in intimate contact with the blood stream and are directly exposed to foreign substances introduced into the circulating blood. Thus it is that these cells can take up India ink introduced into the blood whereas the phagocytic cells of the connective tissues generally have little opportunity to take up carbon particles which escape with such difficulty

through the capillary walls. Other cells like those which line the sinusoids of the spleen also have direct access to ink or dyes which may be present in the circulating blood. The endothelial cells of certain capillaries in the cortex of the adrenal and in the bone marrow also have phagocytic properties and may take up vital dyes, but the endothelial lining of blood vessels elsewhere seems to be almost entirely devoid of ability to retain these foreign substances.

Aside from these specific and well localized phagocytic types of endothelium, one finds in the connective tissues themselves enormous numbers of cells which are capable of taking up and storing in granular form a great variety of foreign substances which are sufficiently diffusible to pass through the capillary walls. A vast group of workers have shown that these cells of the connective tissues vary considerably in their morphology and there is still much uncertainty regarding the derivation of many of them.

Although many of the cells of connective tissues show little avidity toward acid colloidal dyes, still the majority can be stained under proper conditions of dosage and length of staining. Mast cells, lymphocytes and ordinary leukocytes show almost no tendency to take up these "acid dyes," but in the connective tissues are a vast number of "macrophages," "tissue phagocytes," "histiocytes," "clasmatocytes," or "resting wandering cells" as they are variously called, and these cells are highly phagocytic. They will take up particles of carbon or other finely granular material if this is injected directly into the tissues, but even more interesting is their capacity to take up various dyestuffs which are injected locally or which diffuse from the blood stream into the tissue fluids. In building up these intracellular granules, the dye is taken from solution and is greatly concentrated within the cell. It is obvious that the dye is not held there in simple solution, for the concentration is clearly many times that of a similar globule of a saturated solution of the dye. In the case of a few of the acid dyes, needle-like crystals may be formed but in no case is the exact nature of this concentrating mechanism understood. The literature of this question has been reviewed by Möllendorff (6). It remains an open question whether this process of dye storage differs fundamentally from the phagocytosis of finely particulate matter by these same cells. The former has been referred to as "dypsocytosis" (9) but this term has not gained wide acceptance. In the present experiments we have used the term "phagocytosis" to cover both. We have also used the term "vital staining" to refer to the dye storage, despite the fact that the word "staining" would seem to infer a reaction between dye and protoplasmic granules or other cytoplasmic constituents—a point still very much in dispute in the case of the "acid" dyestuffs.

Aside from these macrophages which are capable of ameboid motion, the fibroblasts of the connective tissues show an interesting behavior toward these acid vital dyes. Concerned as they are with the formation and maintenance of the fibers which bind the parts together, these cells have a much underdeveloped ability to take up foreign substances from the surrounding fluids. The faint

granular storage of lithium carmine by these cells is well shown in the plates of Ribbert (10) and Kiyono (11). Goldmann (2) showed that they will take up pyrrol blue. Evans and Scott (9) have placed particular emphasis upon the fact that they take up the dye much later than do the macrophages, and they have shown that granules do not appear in the fibroblasts until the animal has received large quantities of the dye. This sharp distinction in reaction has led Evans and Scott to set them aside as a distinct physiological class of cells.

These morphological details give us much valuable information concerning important physiological reactions of the tissues—reactions which would pass unobserved were it not that the tinctorial properties of these dyestuffs make their recognition easy. There is every reason to suspect that many products of normal metabolism in the body may be distributed in analogous ways, though they lack the color properties necessary for their demonstration.

It is to be regretted that the morphological studies with dyestuffs have always been so purely qualitative. It is most desirable that we know more about their distribution in the tissues from a quantitative point of view. Unfortunately histological methods are almost purely qualitative and chemical extraction of ingested dyestuffs is beset with serious difficulties.

At the present state of our knowledge it is perhaps as well that we restrict our quantitative studies to the phagocytic response of the body tissues as a whole. Adequate means for such studies exist, for, with certain restrictions (12), the rate at which a dye leaves the circulating blood stream is a measure of this tissue response, representing as it does the sum total of the absorptive power of the elastic tissue and of all of the "macrophages" and other phagocytic cells previously mentioned. The reason that this quantitative mode of attack has been neither popular nor successful in the past must be that many dyestuffs are not well suited for such studies. Many of them leave the blood stream in large part before there has been time for them to be thoroughly mixed with the circulating blood. Under these circumstances efforts to follow the rate of elimination from the blood stream must necessarily fail. Other substances, like India ink and many dyestuffs are precipitated by the body fluids, and the particles are mechanically caught and held for a time in certain of the capillaries. Obviously, here too, the rate of disappearance from the blood is no criterion of the phagocytic response of the tissue. With other dyes the elimination through the urine or the bile is so rapid that the tissues are afforded little opportunity to display their phagocytic properties, and here again the elimination rate gives none of the desired information concerning tissue absorption. Fortunately, certain dyestuffs are relatively free from these defects, and from this group we have selected two, brilliant vital red and Niagara sky blue, as being excellent representatives. A number of the physiological studies have been reported in the previous papers of this series. The results of those experiments will be reviewed in the light of the morphological studies about to be reported.

Methods

Many of the preliminary studies were made on rabbits, but typical experiments of all classes were made on dogs also, and these latter experiments are of particular interest because of the close relationship existing between them and the physiological experiments on distribution and elimination of dye and ink reported in previous papers of this series. In the case of both dogs and rabbits the ink was always given intravenously, but the rabbits in most cases received the dye into the peritoneal cavity, from which it is rapidly absorbed and carried with the circulating fluids to all parts of the body. The dyes and ink were given to the dogs by the intravenous method. The dogs received the regular mixed diet of hospital table scraps; the rabbits received hay, oats and cabbage.

The distribution of India ink can be studied to advantage in fixed tissues imbedded in paraffin and cut and stained by any of the ordinary methods. The two dyes, brilliant vital red and Niagara sky blue, are stored in granular form in many phagocytic cells scattered throughout the body, but the dye appears to be rather loosely fixed within the cell and after death it tends to diffuse, especially if the tissues are placed in any of the solutions ordinarily used in fixing and imbedding. Niagara sky blue suffers from this defect somewhat less than does brilliant vital red but in both cases the results in fixed tissues are quite unsatisfactory, and clear-cut pictures are to be obtained only by the study of freshly drawn films of tissues, teased preparations and fresh frozen sections of unfixed tissue. Even in these cases it is best to avoid adding water or saline to the preparation, and it is well to study the section immediately, without the addition of fluid or cover glass, though addition of glycerine and a cover-glass sometimes gives very satisfactory results. A drop of immersion oil may be placed directly upon preparations of omentum or mesentery and the tissue thus made available for study with the oil immersion lens.

It is desirable that several workers be concerned in making the fresh tissue preparations, for much valuable time may be saved and a maximum number of preparations may be prepared and examined before the cells die and the dye begins to diffuse away. Even so, it is rarely possible to make a detailed study of every organ while the tissues are fresh and much of the information is necessarily gained by piecing together the results of several similar experiments.

EXPERIMENTAL

The dye granules formed within the cytoplasm of living cells present great variations in size, shape and arrangement depending upon the dye employed and upon the amount of dye administered and upon the rapidity with which the staining is brought about. Much of the earlier work has been reviewed by Möllendorff (6). The magnitude and complexity of this subject is well brought out in an excellent monograph by Evans and Scott (9). Their experiments cover a wide territory

and they have formed certain general conclusions regarding the effect of dosage and the duration of the treatment with dye. It is our hope that much detail still lacking may be filled in and that it may be possible to bring such morphological studies more closely into relation with the physiological aspects of dye storage. We feel that the morphological studies gain greatly in significance if carried out in conjunction with quantitative measurements of the rate at which the cells remove the dye from lymph or plasma. Our previous studies (12, 13) on the rate at which brilliant vital red and Niagara sky blue pass out of the blood stream into the tissues have not been extended in detail to other dyes, and for this reason it seemed desirable that we confine our microscopic studies to these two dyes.

We have found interesting cytological details which vary with the amount of dye and with the way in which it is given. Much of this work must be presented at a later time. At present we can consider only those findings most directly related to our previous studies on the rate at which these dyes leave the circulating blood.

Experiment 22. Moderate Staining of Dog with Brilliant Vital Red.

Dog 28–266. Brown terrier, 8.2 kg.

May 20–23 inclusive, 6 cc. 2 per cent brilliant vital red daily. Eight more on 24th and 6 cc. on morning of May 25. Animal killed with ether several hours later. Tissues moderately stained. Skin and connective tissues delicate pink. Lungs almost free of dye. Aorta moderately stained. Liver dark red. Bile thick and deep cherry red. Lymphatics at hilus of liver distended with clear reddish lymph. Spleen not enlarged but rather red and firm. Cortex of kidney moderately red. Medulla pale pink. Urine from bladder clear and yellow. Moderate staining of ileo-cecal lymph glands. Central two-thirds of each is bright red. Choroid plexus dark red, but meninges and brain unstained.

Microscopically, a bit of omentum spread out on a slide shows enormous numbers of macrophages. Careful inspection shows that they contain very large numbers of extremely fine pink granules. No conspicuous large clumps of dye in any of these cells. All these cells are stained very much alike and all have round or slightly oval nuclei. The cell bodies are irregularly globular. No dye in fibroblasts. No dye in endothelium of vessels, though elastic tissue of walls of certain vessels is pale pink. Many Kupffer cells contain moderate numbers of dye granules. No large dye clumps. Many convoluted tubules of kidney contain numerous fine dye granules in epithelial cells but no granules in part of cell next to lumen. Many granules about the nuclei or at the basal side of the cells. Hypophysis faintly pink, and frozen sections show numerous irregular triangular cells containing fine dye granules. They appear to be between the epithelial cells. No dye seen in the latter. Epithelial cells of choroid plexus contain numerous dye granules which are extremely fine and uniform in size. A few cells, apparently macrophages, between these cells contain more abundant dye.

Experiment 23. Heavy Staining of Dog with Brilliant Vital Red.

Dog 28–93. Fat female poodle, 8.0 kg.

April 24–May 1, 24 cc. 2 per cent brilliant vital red daily. Dog somewhat weak on last day. Centrifuged plasma obtained five minutes after last dye injection contained 1030 mg. dye per liter; six hours later 830 mg. Dog then killed with ether. Connective tissues deep red, especially the fascia of the muscles. Liver, spleen and kidneys deep red. Aorta and other large vessels deeply stained. Microscopically, elastic tissue of aorta is pink. Bits of mesentery and omentum spread out on slides show many macrophages varying somewhat in size, the largest ones being about 30–40 micra in diameter. The granules of dye which they contain are decidedly irregular and angular and variable in size. No crystal formation in any of the cells. Apparently the central portions of certain of the larger granules are a trifle paler than the peripheral portions. Epithelial cells of liver contain no dye but the Kupffer cells are much more numerous than normal and contain many large and small dye granules. Many small angular cells, apparently endothelial cells, in adrenals contain dye granules but no dye seen in the epithelial cells. Fibroblasts in connective tissue films and in mesentery and omentum have distinct oval nuclei and there are many small spherical uniform dye granules in the cytoplasm. Such granules are present also in the ramifying processes of these cells. This staining of fibroblasts is very faint.

Experiment 24. Staining of Dog with Brilliant Vital Red. Examination of Connective Tissue Films from Thigh.

Dog 28–281. Short-haired brown adult mongrel, 12.6 kg.

July 1–4 inclusive, 15 cc. 2 per cent brilliant vital red injected daily. Preceding the injection on July 4 an area on inner side of hind leg was shaved and with aid of novocain a small incision in skin was made and bits of connective tissue removed from between muscles. Film preparations covered with glycerine and coverglass were examined with oil immersion lens. There are moderate numbers of dye-laden cells, most being rather thin and somewhat elongated, but not so much so as fibroblasts. Some cells contain more dye than others. All granules are rather uniform in size and none are extremely large. Most cells contain between 30 and 50 granules each.

July 6–9 inclusive, 15 cc. 2 per cent brilliant vital red injected daily. Films again examined on July 10. Preparations rather variable but rather clearly there are more dye-laden cells than on July 4. The enlargement of individual cells is very stiking, most of them being several times as large as on July 4, and they are more rounded and many of them are much more than 30 micra in diameter. The dye granules are still uniform in size, though posssibly somewhat larger. The increase in number of granules per cell is very striking. Many contain well over 100, and some more than 150. The intensity of dye in individual granules appears unaltered. No change in shape of dye granules.

No more dye injections made, but during following days skin and mucous membranes became more pale. Films again made on July 17. There appear to be

as many dye-laden macrophages as before, and dye-containing fibroblasts are possibly more numerous. They contain many fine granules of dye but these granules are quite small. The macrophages appear unaltered in size and the granules are certainly not less numerous but perhaps slightly smaller. Very clearly the granules vary more in size and many macrophages contain 1–3 large irregular granules or clumps of dye. No evidence of change in shade or quality of color of dye granules, though a few appear a little paler than before.

The general distribution of brilliant vital red is rather similar to that of dyes studied by Ribbert (10), Goldmann (2), Schulemann (3), Kiyono (5) and others. Experiment 22 will serve to illustrate the findings where the tissues are but moderately stained with this dye. In contrast to this experiment the tissues in experiment 23 were heavily stained. This was quite evident on gross inspection of various organs and tissues.

Histological study shows in each case the formation of dye granules in widely scattered cells. Most brilliant deposits are seen in the Kupffer cells in the liver, in the sinus endothelium of the spleen and in the phagocytes of the splenic pulp. We recognize also the well-known deposits in the sinus endothelium of lymph nodes, in the endothelium of the capillaries in the adrenal cortex, and above all the deposits of dye in the macrophages scattered throughout the connective tissues generally. As Evans and Scott have shown, the fibroblasts take up dye much more feebly than do the macrophages, but in experiment 23 so much dye had been injected that the fibroblasts show slight though definite granules of dye. This participation on the part of new cell types helps explain the ability of the body to handle ever increasing amounts of dye. There is also reason to believe that large or often repeated smaller doses of dye stimulate proliferation on the part of the more active types of phagocytes.

Investigators have repeatedly stressed the fact that with chronic vital staining one sees increased numbers of Kupffer cells in the liver sinusoids. This is entirely in accord with our own observations. It is difficult to estimate an increase in the number of macrophages in the connective tissues, for they are always irregularly scattered and it is difficult to get representative preparations. Nevertheless it does seem that some such increase does occur. Cell proliferation on the part of phagocytes represents an adaptation in time of

stress, and the dye storage by fibroblasts must also help to rid the circulation of these foreign materials.

We must recognize that the cell itself has remarkable powers of adaptation. Thus, the heavily stained animal (experiment 23) has much larger dye granules in the cells than does one which is feebly stained (experiment 22). Along with this increase in size of granules it appears that the individual cells increase somewhat in size.

We must confess that frozen sections and teased preparations of the internal organs are not entirely satisfactory for the study of fine cytological details, especially in the case of dyes which so readily diffuse out of the cells after death. It is much easier to get satisfactory preparations from the subcutaneous tissues of the thigh. Evans and Scott (9) have made beautiful films of such tissue and we can agree with them that with proper precautions one can make out the finest details regarding the shape, size and even the number of granules within the cells. Our observations on these tissues show that cell granules may increase markedly in number during the earlier stages of staining and as representative of these observations we may cite experiment 24. We note that in this experiment the increase in size of the macrophages is quite evident as soon as they begin to show considerable deposits of dye granules. In fact it would seem that the increase in size is almost proportional to the increase in the amount of dye within the cell. The result is that the dye granules seem to be no more closely packed than they were during the earlier stages of staining. It is noteworthy that in this experiment there is very clear evidence that the dye granules have increased greatly in number. It would seem that after certain granules had reached a certain size, new foci are formed, but our inability to find numbers of these newer and smaller foci would suggest that relatively few active centers of growth occur at any one time but that these centers grow rapidly and newer ones are then formed.

This experiment appears to be out of agreement with experiments 22 and 23 just cited. These pointed to the conclusion that many small granules are present at first and that they all enlarge to reach the size seen in the more heavily stained animal. We are unable to state the factors which determine the growth by accretion on the one hand or the growth by new granule formation on the other. We believe that both processes occur. Perhaps each mode is character-

istic of certain cell types, but slight differences in mode of administering the dye must also be considered. The importance of the time factor is evident from experiment 24 just mentioned, for after the dye injections had been discontinued for one week we noted that each macrophage contained several large irregular granules as well as many smaller ones. There is rather obviously a decrease in the staining of the tissue with the passage of time and this can be seen in gross as well as from the microscopic study. The granules do not seem to have decreased greatly in number, though many of them appear smaller. It would seem that some had grown at the expense of others or that certain of the granules had fused to form these larger masses. We are carrying out further experiments in an attempt to clear up these and other closely related points. It is felt that such data gain greatly in significance if carried on together with studies of the rate at which the dye leaves the blood stream, for these latter data give us much important information concerning the functional activities from a quantitative point of view.

Of great interest from the physiological point of view is the distribution of the dye in organs such as liver and kidneys whose activities in secreting various dyestuffs is common knowledge. Brilliant vital red belongs to that group of dyestuffs which passes into the urine only in small amounts. Unless very large amounts of dye are injected the small amounts of dye present in the urine are so overshadowed by the normal urinary pigments that they would not be recognized by gross inspection, but if the urine be passed several times through filter paper the latter will be found to be stained a delicate pink color. This staining is dependent upon the selective adsorption of dye by cellulose.

That the kidneys secrete so little dye is distinctly surprising in view of the fact that certain of the convoluted tubules contain such beautiful and conspicuous granules of dye. On the other hand, the epithelial cells of the liver contain no granules of brilliant vital red and yet the dye passes into the bile in considerable quantities (15–30 per cent in 24 hours). These facts support the numerous investigations of recent years which are leading to the point of view that granule formation in the epithelium of glands is a process which may be quite unrelated to the question of whether or not the gland can excrete that particular dye. Very obviously the dye can pass through the epithelial cells without forming any granules in the cytoplasm. In fact a dye may gain passage quite rapidly, so that the amount in the cytoplasm at any one time may never be enough to produce even a noticeable diffuse staining.

In general the distribution of Niagara sky blue in the body is quite similar to that described for brilliant vital red. A few minor differences are seen. From the curves given in a previous paper (14) it is evident that during the first few hours Niagara sky blue leaves the blood stream distinctly more rapidly than does brilliant vital red, though an equilibrium partition between tissues and body fluids seems to be reached rather sooner, and at a time when relatively large amounts of the dye still remain in circulation. We have repeatedly observed that animals stained with the blue dye show conspicuous staining of skin and mucous membranes many days longer than do dogs stained with similar amounts of brilliant vital red. We have no way of knowing whether this is associated with differences in elimination from the body, for unfortunately the blue dye unlike the red one is partially decolorized on admixture with bile, so that it is impossible to compare the elimination of the two in fistula dogs. The blue dye passes into the urine somewhat more readily than does the red one, but the total lost in that way is not great.

The ability of the body to decolorize many dyes is common knowledge. Some are decolorized almost immediately. Teploff (15) shows that carmine granules within the cells gradually change in shade and become yellow or black in the course of weeks, indicating slow chemical transformation. We have studied connective tissue films removed from animals at intervals following a series of injections with brilliant vital red, but we have been unable to see any change in the shade of brilliant vital red with the passage of time. We have not yet made microscopic examination of the internal organs during this stage of regression, and the possibility remains that some of the dye may undergo such change in these organs. We do know that any decolorization which does occur is not great, for it is often possible to recover 50 to 85 per cent of the dye in the bile in the course of 5 or 6 days. After that the bile may still show a reddish tint, but the amount of dye present is too small to be measured in the presence of the bile pigments. We believe that most, if not all, of the dye gradually passes from the tissues back into the blood, from which it is removed and eliminated by the liver. It is only in a few places as in the centers of the lymph nodes that the dye is retained over a period of months. Certain of these cells seem to hold to the dye with the greatest tenacity, though in time they too lose their dye.

Experiment 25. Staining of Dog with Mixture of Brilliant Vital Red and Niagara Sky Blue.

Dog 27–107. Female mongrel, 9.0 kg.

Injection mixture made by mixing equal parts of 1.5 per cent brilliant vital red and 1.5 per cent Niagara sky blue. Five daily intravenous injections of 14 cc. each. No dye on 6th day. Animal killed with ether on 7th. No toxicity. Tissues uniformly purple. Endocardium of heart and walls of larger vessels bluish-purple. Lungs faintly stained, and in fibrous tissue frame-work are seen irregular cells containing purple granules of dye. Many phagocytic cells in splenic pulp. They contain reddish-purple dye granules. Liver purple. Kupffer cells contain purple granules. Epithelial cells free of dye. Epithelial cells of kidney contain many dark purple dye granules. No pure red or blue granules. Macrophages in areolar tissues and in mesentery also contain only purple granules.

In conclusion, no cells anywhere contain either pure red or blue granules though all granules in spleen were reddish-purple while those in liver and kidneys were bluish-purple.

Experiment 26. Staining of Rabbit with Mixture of Brilliant Vital Red and Niagara Sky Blue.

Female rabbit, 3.1 kg.

Injection mixture made by mixing equal parts of 1.5 per cent Niagara sky blue and 1.5 per cent brilliant vital red. On first day 3 cc. injected. None on second day, but on following days, 5th, 6th, 7th, 7 cc. respectively were given. No toxicity. On following day animal killed with ether. Skin and mucous membrane quite purple. Spleen, liver, and kidneys also. Macrophages in connective tissue of thigh and in omentum show many discrete reddish-purple granules. Some of those in phagocytes of spleen are purplish-blue; others bluish-purple. Epithelial cells of liver contain no dye granules. Kupffer cells contain many irregular purple granules. Epithelial cells of convoluted tubules of kidney are bluish-purple.

Data of great interest are to be obtained by administering two different dyes to the same animal. It is well that they be of widely different color in order that one may recognize each in the cells. We have already shown (16) that purple plasma containing a mixture of red and blue dye may be analyzed in the spectrophotometer and the amount of each dye present in the mixture determined, and with this method it was possible to follow the elimination of each dye from the blood stream even though both dyes were present together in the plasma. It was shown (14) that each dye is finally distributed between plasma and tissues in a way characteristic of that particular dye and at a rate quite independent of the presence of the other dye in the plasma and

tissues. In certain of these physiological studies the two dyes were injected simultaneously. In others a period of intensive staining with one dye preceded the injection of the second. Histological studies of various tissues are necessary as a basis for any attempt to interpret these results.

Dog 27–107 (experiment 25) received 105 mg. of each of the two dyes daily over a period of 5 days. Gross inspection of the skin and mucous membranes showed progressively increasing purple coloration, and the animal when killed with ether showed similar changes in the internal organs. It is of interest that the aorta, and larger blood vessels generally, show a distinct excess of the bluish coloration, but there is an element of red also. This is quite in agreement with our previous observation that elastic tissue shows greater affinity for Niagara sky blue than for brilliant vital red. Microscopical examination of the aorta always confirms the gross findings and shows that the color is due to diffuse staining of the elastic fibers. Other tissues show the phagocytic reaction in a very beautiful manner. The Kupffer cells of the liver, the endothelial cells and pulp cells of the spleen, the phagocytic cells of lymph nodes and the macrophages of the connective tissues contain beautiful deposits of dyes. We were rather surprised to find that almost without exception the granules within the cells contained a mixture of the two dyes, and as a result were purple.

In one or two experiments of this sort we have found an occasional granule which appeared to be either pure red or pure blue, but this is the exception. Experiments of this sort demonstrate that these two dyes are distributed within the body in almost identically the same manner. The existence of mixed granules indicates that even within the substance of the cell the protoplasm does not discriminate between the two. Experiment 26 shows practically identical results when the two dyes are given simultaneously to a rabbit. We have other experiments with dogs and with rabbits and all of them are in agreement.

Experiment 27. Staining of Dog with Niagara Sky Blue Followed by Staining with Brilliant Vital Red.

Young female mongrel dog, 5.5 kg. December 16, 20 cc. 2 per cent Niagara sky blue intravenously. Similar injections on following day. December 18th and again on 19th, 20 cc. 2 per cent brilliant vital red intravenously. No toxicity. Animal killed with ether on December 20th. Skin purple. Subcutaneous con-

nective tissues bluish-purple. Lungs very slightly stained. Endocardium of heart dark purple. Aorta purple, and microscopically elastic fibers in it are diffusely stained more blue than red. Many fibers from tough subperitoneal tissue are pure blue, others bluish-purple. Spleen purple. Malpighian bodies paler and grayer than pulp. Microscopically many large irregular cells contain granules varying greatly in size and shape. Many cells contain more red dye than blue and a few contain red ones only. A few small cells contain only blue granules. Many cells contain both types but no purple granules found. Liver deep bluish-purple. Epithelial cells contain no dye granules but Kupffer cells are very large and numerous. They contain many granules of both colors but blue ones are more numerous. A very small number of cells contain only red ones and these are rather less irregular in shape than the rest. No purple granules in any of the cells. Bile deep orange color. Kidney cortex stained but medulla pale. Microscopically many granules in cells of convoluted tubules. They vary greatly in size and shape. They are red, triangular masses and granules and rods with projecting knobs, some of these knobs being of a different color than the main mass, and often granules of different color are in contact but there are no purple granules. Urine olive color, clear. Mesenteric lymph nodes deep purple. Many phagocytes contain both dyes separately but no mixed granules. Very few cells contain only one type of dye. Mesentery contains many cells with granules more or less spherical. Red and blue granules about equally numerous. In places the two types of granules seem to be loosely in contact but in no place are they fused and in no case is a zone of one dye layered about the other.

Experiment 28. Rabbit Stained with Brilliant Vital Red Followed by Staining with Niagara Sky Blue.

Male rabbit, 2.7 kg.

Five daily intravenous injections of 3 cc. brilliant vital red (1.5 per cent). Two days later 3 daily injections of 4 cc. each of 1.5 per cent Niagara sky blue. Animal killed with ether forty-eight hours later. Mucous membranes, kidneys, liver, spleen quite purple. In liver and spleen there is a rather less amount of both dyes than usual but red dye is more abundantly present than blue. Some Kupffer cells contain only red dye but many contain granules of each. No purple granules. No granules in epithelial cells of liver. Epithelial cells of convoluted tubules of kidney contain red and blue granules but no purple ones. In general red granules are more in evidence in the part of the cells most distant from the lumen, while in the areas bordering lumen blue granules predominate. Beautiful double segregation of dye in macrophages in connective tissues and mesentery. Very few cells contain one type of dye only. No purple granules anywhere. The blue dye is rather more in evidence in these cells than in Kupffer cells of liver and phagocytes of spleen.

A very beautiful contrast to experiments of this sort is afforded if the two dyes be injected *in successive periods*. Thus, in experiment 27 we

gave 2 daily intravenous injections of 20 cc. of 2 per cent Niagara sky blue to a dog; this was followed by 2 daily injections of brilliant vital red. The tissues, at first blue, now took on an intense purple color. At autopsy the gross appearance of the organs was identical to that when the two dyes had been given simultaneously, but the microscopical picture was surprisingly different. There was little difference as regards the diffuse staining of the elastic fibers, but we were unprepared to find that in no instance were the two dyes associated to form purple granules. Quite in contrast, the phagocytic cells scattered throughout the body contained innumerable red granules as well as innumerable pure blue granules. In almost every case these two types of granules were clearly distinct from each other though here and there granules of the two types appear to lie in contact, but neither were they fused to form mixed purple granules nor was the red dye layered about the blue dye granules as one might expect from the mode of administration. Further experiments of this sort in dogs confirmed these results. In a number of experiments on rabbits similar results were obtained. The protocol of experiment 28 is typical.

These observations raise a number of important considerations regarding the mechanism by which dye granules are formed within cells and we shall return to this subject later. We may recall here our previous inability (14) to "block" the tissues with one dye against the entrance of another. Without detailed microscopic study one might have suspected that the two dyes were taken up by two entirely separate and distinct sets of phagocytic cells, but we have just seen that such is very clearly not the case, no matter whether the dyes are given simultaneously or in sequence. It is now obvious that in the case of these two dyes the cells may contain large quantities of one dye and still fully retain their ability to take up another at the normal rate. This is all the more remarkable when we realize that these two dyes are rather closely related in their chemical properties, and with the exception of their color in most of their physical properties also. If "blockade" is ever to be obtained, one would expect it in the case of such closely similar substances which are taken up by identically the same cells and, indeed, under certain circumstances brought intimately together within the same granules. Whatever the mechanism of the segregation of dye may be, it is at least clear that

the cells react toward each dye in a way which is independent of how much of the other dye may be within the cells, or in the surrounding fluids.

In this connection we wish to emphasize our observations (14) that the amount of either dye which may be taken up is related to the concentration of that dye in the surrounding fluids. Very evidently, each dye establishes its own partition between cells and fluids independently of the presence of the other. It is tempting to compare the partition of dyes between cells and fluids to the phenomenon of partition well known to physical chemists, where a substance may be distributed between two immiscible fluids and the relative amount in each layer may be very slightly or not all influenced by the presence of other similar substances which may undergo partition according to laws peculiar to themselves. We recognize the dangers of such analogies and we do not wish to pursue the comparison further, but it is significant that in the physical as well as in the biological phenomena several substances may undergo partition largely independently of each other.

We have injected dogs with Higgins' "American" India ink and have studied the influence of this procedure upon the rate at which brilliant vital red will leave the blood stream. A report already made (17) shows that in such animals there is a very marked retention of dye in the circulating blood, and at first we had thought that the ink previously given had "blocked" the phagocytic cells or had reduced their ability to take up dye. Studies of this sort made on bile fistula dogs showed that a different explanation is much more likely, for these experiments showed that during the period of observation almost no dye is excreted into the bile if the dog had received ink at the beginning of the experiment. Control experiments showed that normally such dogs would secrete rather large quantities of dye during the four-day period of observation. The failure of the ink-treated dogs to excrete the ordinary amount of dye through the liver seemed to account quite adequately for the longer retention of the dye in the circulating blood. Under these circumstances the dye is largely retained in the body, and throughout the course of the experiment more dye was present both in plasma and in tissues. Had we remained in ignorance of this altered liver excretion we might have fallen into the error of assuming a blockade on the part of the phagocytes. It seems quite possible that others have made such mistakes when studying "blockade." Many dyes are normally eliminated by liver or kidney much more rapidly than are brilliant vital red or Niagara sky blue, and, with those dyes especially, any disturbance in the excretory power of liver or kidney would pro-

duce even more striking retention in the blood stream. Any such retention must never be credited to defective phagocytosis until all of these other factors have been given the most careful attention. We feel that experiments indicating the presence of a "blockade" should be controlled by the study of elimination through liver and through kidneys.

We have made some little progress in determining how ink inhibits the excretion of the dye by the liver, but the experiments are not yet complete. Preliminary experiments indicate that some component of the ink other than the carbon is responsible, for experiment shows that the carbon can be removed from India ink without robbing the latter of its inhibitory power. Furthermore, finely ground graphite suspensions were shown (17) to be inert. Were it not for these experiments we might conclude that the failure of the liver to excrete dye was in some way associated with the fact that the liver comes to contain large quantities of carbon, deposited largely within the Kupffer cells of the liver sinusoids.

From a somewhat more restricted point of view, a study of the distribution of ink particles in the body offers much of interest, for these details are intimately concerned with the entire problem of phagocytosis and blockade of phagocytes. The distribution of the carbon particles in the body differs from that of our vital dyes in several interesting and fundamental respects, and it seems desirable that substances so radically different from the vital dyes should be studied in conjunction with the latter in order that we may learn the physiological response of the phagocytes to each and detect and differences which exist.

For the distribution in the tissues of ink or graphite suspensions introduced into the blood stream we refer particularly to the articles of Goldmann (2, 18), Kiyono (11), Nagao (19), Foot (20), Wislocki (21), Brickner (22), Palmer and Higgins (23), Higgins and Murphy (24).

DISCUSSION

Most recent workers agree that there are important differences between the behavior in living tissues of "acid" and of "basic" dyes. Perhaps the difference is associated with physico-chemical reactions involving the positively charged colored ion of "basic" dyes and the negatively charged colored ion of "acid" dyes. Möllendorff (6) and Herzfeld (25) have stressed the idea that the "basic" dyes unite with electro-positive structures normally existent in many cells. They have also stressed the view that the "acid" dyes do not unite with preexisting structures within the cell, but that their storage is a process closely related to the phagocytosis of larger particles, if not identical with it. It is common knowledge that these two classes of dyestuffs may enter the same individual cells, but in general the distribution in the body and the details of staining are profoundly

different. We draw attention to these facts lest our experiments with the "acid" dyes, brilliant vital red and Niagara sky blue, be confused with known facts obtained with basic dyes.

We do not propose to discuss the many differences which exist between the various "acid" dyes.

Special reference is made to the comparative studies of Schulemann (3, 26). He and other workers have reported experiments involving double staining with acid dyes. It would lead too far to discuss in any detail the confused literature dealing with the subject. We may refer to Steckelmacher (27), Möllendorff (6), Aschoff (7), and Börner-Patzelt, Gödel, and Standenath (8) for literature. No really systematic study of the subject has been undertaken and it is no wonder that confusion prevails when the literature consists largely of isolated observations with the widest variation in dye, in species of animal and in mode of administration. Among the best controlled experiments on this subject are those of Steckelmacher (27). With the aid of lithium carmine and tolidine blue he observed double staining in liver and kidneys. His work on the frog and ours on mammals would seem to indicate that the mode of administration is of the greatest importance in the formation of mixed granules. This point of view was recognized by Schulemann (3) in certain of his earlier studies with trypan blue and vital new red. Separate injection of these two dyes gave him separate blue and red granules side by side within the cell. He encountered difficulty in producing purple granules when the two dyes were injected simultaneously, but he pointed out that vital new red is much more diffusible than trypan blue and that in all likelihood most of it is already anchored within the cells before much of the blue dye has had time to enter the cells. The result is as though the two dyes had been injected at separate intervals, and for this reason Schulemann was not surprised at his failure to find purple granules. Unfortunately he did not test out two dyes which were more nearly alike in regard to speed of penetration.

Our experiments as well as those of Schulemann and Steckelmacher all represent a rather acute type of staining. In no case did the process of staining extend over a period longer than a few days. In our own case this was felt desirable in order that the experiments might serve as controls on our previous measurements (12, 13) of the rate at which these dyes are taken up from the circulating blood.

Evans and Scott (9) report studies on vital staining in which the dyes were administered over a period of several weeks. They were particularly interested in the crystals of trypan blue which may form in the cytoplasm in these chronic types of experiments. They report finding separate deposits of trypan blue and T 148 even when the two dyes were given simultaneously, and when given

separately they found purplish granules as well as red and blue ones. The influence of the longer administration should be studied to compare the results with experiments like ours and Steckelmacher's. Admittedly the two dyes used by Evans and Scott differ considerably from each other and this must be kept in mind when attempting to correlate their results with ours.

The method of double dye injection should be of great service as an aid in the study of just how dye granules are built up within the cell. The difference in color makes it possible to follow the sequence of events. Our own experiments suggest that granules may be built up in several ways—either a few at a time, or many simultaneously and at nearly equal rate. Many more data must be secured before we can assign proper importance to the various factors which determine these finer cytological details. It should be evident that such information should be developed as a basis for physiological concepts and for physiological experiments on distribution of dye between tissues and surrounding fluids.

The experiments of a previous paper (12) showed that when large amounts of dye are given all at once or over a period of several days there is a well marked delay in the rate at which the dye leaves the blood stream. It was shown that *a priori* this may be due to a sluggish response on the part of dye-laden phagocytes, or to inefficient excretion of dye into the bile. Actual experimental study of these two possibilities showed that there is undoubtedly defective liver excretion when large doses of dye are given to the animal. In the course of 24 hours only 10 or 15 per cent of the dye may reach the bile, in contrast to 25–35 per cent when smaller doses are given. We expressed our belief that the longer retention in the blood stream may be due to this defective liver excretion alone, and not to faulty phagocytic power on the part of the tissues. If this is so we must accept the view that in the range of dosage studied, the distribution ratio of dye between plasma and tissues is constant, regardless of the dose employed. We may well be surprised at such a conclusion. It would seem to imply a vast reserve of phagocytic power not previously suspected. If we choose to believe that the tissues adapt themselves to the increased burden of larger dosage, we must also admit that this adaptation is almost instantaneous, for there seems to be no lag in the response when large doses of dye are injected or when a second dye is given simultaneously with the first (12).

Our experiments just outlined suggest a number of methods by which the tissues may adapt themselves. The increase in the number of phagocytes is one which we have observed, and this is commonly cited by others. We may also point out that increase in the size of the cell may help it to accommodate more dye without any loss of efficiency whatever. We may also call attention to the fact that with large dosage new types of cells, such as the fibroblast, take up part of the dye. This spread of the process of phagocytosis to an ever-widening group of cells may be of no little importance in maintaining the powers of the tissues to take up the large quantities of dye forced upon them. The existence of such compensating processes may cause less surprise than the fact that the process of compensation seems to be so prompt, with the result that we cannot even momentarily cause embarrassment to the phagocytic cells. We do not profess to know the relative importance of these various compensating mechanisms in relation to each other; nor may we overlook the possibility that there may be other important ways of maintaining the efficiency of this group of cells.

It may well be that methods may yet be devised to overload these cells. It may even be that refinements in the measurement of liver excretion will show that defective liver excretion will not account for quite all of the retention of dye in the plasma; but even so we cannot possibly deny the fact that the phagocytes have enormous reserve power available, or that they can adapt themselves almost perfectly to added labor within very wide limits.

The data already reported (12, 13) have given us a quantitative concept of the rate at which the body phagocytes will remove certain dyes from the circulating lymph and plasma. Those experiments on healthy living animals furnish information concerning the sum total response of all these cells combined. Details concerning the individual cells and concerning separate groups and classes of these cells are to be obtained by histological study. The attempt to combine the anatomical with the physiological approach has proved most stimulating. The results at present available represent but a beginning. Many data must still be gathered before we can know more than the elementary anatomical facts concerning the building up of dye granules within the cell. In certain instances it would seem that from the very

first the dye is deposited in a vast number of small foci within the cell, and that these tiny granules grow in size as more dye reaches the cell. In other cases it would appear that relatively few foci are active at any one time and that when the granules reach a certain size, new foci of deposition are formed within the cell. The results obtained when two dyes of different colors are given simultaneously or in sequence throw light upon these points.

Intimately connected with the theory of this granular dye deposition is the fact that as the dye deposits increase, the cell may enlarge so much that there may be no more crowding of the granules than in the earlier stages of staining when the cell is smaller and contains fewer granules. Yet, in other cases actual crowding undoubtedly does occur and with certain dyes the granules may agglutinate and needle-like crystals may even form. We believe that differences in dye employed and differences in administration account for many of these discrepancies to be found in the literature. A few of these variations are analyzed in the experiments here presented. All these anatomical details throw light upon our studies of the rate at which the dyes pass out of the plasma into the tissues. Very obviously it is desirable that we know whether the disappearance rate of dye from plasma is associated with increase in size and number of the phagocytic cells.

Our previous experiments have shown that a given dye passes from the surrounding fluids into the cell until a certain equilibrium point is reached. Dye accumulates within the cell and we find that the phagocytic activity comes almost to a standstill long before the fluids have given up all of their color. Control experiments show that dye-free phagocytes will function quite readily in the presence of plasma containing similar amounts of dye. We found also that these phagocytes may contain large amounts of this dyestuff and yet they can take up another closely related dyestuff in quite a normal manner. The histological details regarding the distribution of these two dyes form an interesting and important supplement to those observations.

SUMMARY

Vital staining reactions of brilliant vital red and Niagara sky blue are studied in dogs and in rabbits. Either dye alone is taken up to form red or blue granules within the cytoplasm of macrophages and of certain other cell types.

When the two dyes are injected simultaneously into the blood stream one finds that these cells build up granules which are purple from admixture of the two dyes. When several daily injections of one dye are followed by several daily injections of the other, one finds blue granules and red granules side by side within the cells, but no purple granules are found. This is thought to indicate that the dye is deposited in small foci which are active in a rather transitory way, and that the color of the granule is determined during its formative stage by the type of dye present in the fluids about the cell.

The enlargement of phagocytic cells and the increase in their number with large dosage, or with repeated offerings of the dye, represents a method by which the cells maintain their phagocytic powers at the normal level. Evidence is offered to indicate that these and perhaps other compensatory changes may take place with great rapidity, so that it has been impossible to "block" or even reduce noticeably the ability of these cells to take up additional quantities of dye.

Certain pitfalls in the experimental study of "blockade" are pointed out.

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CONCERNING THE SPECIFICITY AND NATURE OF THE PHENOMENON OF LOCAL SKIN REACTIVITY TO VARIOUS BACTERIAL FILTRATES

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In previous communications (1–10) there was described a new phenomenon of local skin reactivity to culture filtrates of various microörganisms. The reactivity was induced by the injection of a filtrate into the skin of a rabbit. If 24 hours later an intravenous injection of the same filtrate was given to the same rabbit, there appeared an extremely severe hemorrhagic necrosis at the site of the previous injection. The factors determining the local skin reactivity were termed "skin preparatory factors" and those responsible for the local injury following the intravenous injection were called "reacting factors." The object of this paper is to consider the results previously published in the light of certain new data bearing upon the specificity and the nature of this phenomenon.

Effect of Combining Skin Preparatory Factors of One Microörganism and the Reacting Factors of Another Unrelated Microörganism

In early papers (1, 2) a series of experiments were reported in which attempts were made to determine whether the local skin reactivity to *B. typhosus* culture filtrate could be also elicited by culture filtrates of microörganisms other than *B. typhosus*. It was shown that culture filtrates of *Streptococcus pyogenes hemolyticus* and *Streptococcus erysipelatis* failed to substitute for the *B. typhosus* culture filtrate in the phenomenon of local skin reactivity to *B. typhosus*. When, in further studies, attempts were made to elicit this phenomenon by both skin and intravenous injections of filtrates of microörganisms other than *B. typhosus* it was found that a large group of bacteria including the two above mentioned strains of streptococcus failed to elicit it. This showed that the filtrates of certain organisms lacked either skin preparatory or reacting factors or both.

Returning now to the earlier reported experiments with streptococci in which filtrates from these organisms failed to prepare for *B. typhosus* reacting factors the question arose as to whether their inability in the part was due to the lack of non-specific factors or to the lack of specific factors (in this case, *B. typhosus*).

Experiments reported below were made in order to determine whether a culture filtrate of one microörganism could prepare the skin for the injurious effect of another unrelated microörganism, provided the filtrates of both microörganisms were shown to contain potent factors.

Methods.—The mode of injecting and the recording of results were the same as described in previous publications (2, 4, 9). "A_{10}," "A_{11}" and "587" were meningococcus, *B. typhosus* ("T_L" strain) and *B. coli* "agar washings" filtrates, respectively. The technique for preparation of these filtrates has been previously described (6, 7).

Anti-typhoid horse Sera H 4/68 and H/88 were recent bleedings from Horse 4. The mode of immunization of this horse was described in a recent paper (9). Anti-coli horse Serum H/86 was prepared in a similar manner.

Experiment.—Twelve rabbits received each three simultaneous intradermal injections of filtrates of certain microörganisms. The upper right areas of the skin of the abdomen of each of these rabbits were injected with 0.25 cc. of meningococus filtrate ("A_{10}"), the lower right areas with 0.25 cc. of *B. typhosus* filtrate ("A_{11}") and the upper left areas with 0.25 cc. of *B. coli* filtrate ("587"). Twenty-four hours later the rabbits were divided into three groups of four. Each group received intravenously a different filtrate. The rabbits of the first group received per kilo of body weight 1.5 cc. of "A_{10}" previously diluted 1:5, the second group received 1.5 cc. of "A_{11}" previously diluted 1:5 and the third group received 1.5 cc. of "587" previously diluted 1:5. Four hours later there were found three dead rabbits in the first group. The nine surviving rabbits showed severe hemorrhagic necroses ranging in size from 2 x 2.5 to 4 x 4 cm. in all the three prepared areas.

As is seen from this experiment, skin preparatory and reacting factors of various biologically and serologically unrelated microörganisms (*i.e.*, *B. typhosus*, meningococcus, *B. coli*) are able to substitute for one another provided they have the power of eliciting the phenomenon for themselves. Apparently, therefore, filtrates from a variety of cultures are able to induce a state of reactivity in a given area of tissue. Once this state is attained the area becomes vulnerable to potent reacting factors irrespective of the microörganisms from which they are obtained.

Specificity of the Skin Preparatory and Reacting Factors of Various Microörganisms

The object of the work reported in this part of the paper was to determine whether the skin preparatory and reacting factors derived

from various microörganisms differed from each other in their antigenic structure. A series of experiments made with this object in view have been already reported in previous papers (3, 4, 5, 9, 10).

It was found that the *B. typhosus* skin preparatory factors were specifically neutralized by homologous sera. Heterologous sera of a variety of microörganisms, as well as normal sera lacking in spontaneous agglutinins for *B. typhosus*, failed to neutralize these factors. Paratyphoid A and B sera showed some neutralization. Skin preparatory factors derived from microörganisms other than *B. typhosus* were also neutralized specifically by homologous sera.

The *B. typhosus* reacting factors were also neutralized only by homologous sera.* Normal sera free of *B. typhosus* spontaneous agglutinins as well as meningococcus therapeutic sera and certain anti-streptococcus sera failed to neutralize these factors. Meningococcus reacting factors were also neutralized specifically by homologous sera. Again, anti-typhoid immune sera showing a high neutralizing titer for *B. typhosus* reacting factors had no effect upon the meningococcus reacting factors.

It appears, therefore, from what has been stated thus far, that one substance antigenically totally different from another is able to induce a state of reactivity to it. The following experiment affords additional evidence to support this point:

The Effect of Neutralized Reacting Factors upon Areas Prepared with Heterologous Skin Preparatory Factors.—In this experiment there were employed many multiples of the minimal doses of reacting factors of the various bacterial filtrates necessary to elicit reactions in skin sites prepared with given amounts of each of these filtrates. The amounts of sera used were such as were found in previous experiments to give consistent neutralization with these multiples of homologous reacting factors. The experiment was carried out as follows:

Experiment.—Sixteen rabbits each received three simultaneous intradermal injections of various filtrates. The upper right area of the skin of the abdomen of each rabbit was injected with 0.25 cc. of undiluted "A_{11};" the lower right areas were injected with 0.25 cc. of undiluted "A_{10}," and the upper left areas were injected with 0.25 cc. of undiluted "587" filtrate. Twenty-four hours later the rabbits were divided into four groups of four. Each rabbit received a single intravenous injection. The rabbits of the first group received per kilo of body weight 0.5 cc. of "A_{11}" previously diluted 1:2; the second group received 0.5 cc. of

* Sera of the paratyphoid group were not tested.

a mixture of equal parts of "A_{11}" and Serum H 4/68; the third group received 0.5 cc. of "587" previously diluted 1:2, and the fourth group received 0.5 cc. of a mixture of equal parts of "587" and Serum H/86. Four hours after the intravenous injection, the results were as follows: In the first group two rabbits survived. Both showed severe hemorrhagic necrosis in all the three prepared areas. In the second group all the rabbits survived and showed no reactions. In the third group all the rabbits survived. Three rabbits showed severe reactions in the three areas. One rabbit showed no reactions. In the fourth group all the rabbits remained alive and no reactions were seen. A summary of the results is given in Table I.

TABLE I

The Effect of Neutralized Reacting Factors upon the Areas Prepared with Heterologous Skin Preparatory Factors

Injections into the skin of the abdomen			Intravenous dose per kilo	Total of rabbits tested	Rabbits showing reactions in three prepared skin sites	Number of rabbits showing no reactions in prepared skin sites	Number of deaths
Upper right areas	Lower right areas	Upper left areas					
0.25 cc. of "A_{11}" (*B. typhosus* filtrate)	0.25 cc. of "A_{10}" (meningococcus filtrate)	0.25 cc. of "587" (*B. coli* filtrate)	0.5 cc. of "A_{11}" dil.-1:2	4	2	0	2
"	"	"	0.5 cc. of mixture of equal parts of "A_{11}" and Serum H 4/68	4	0	4	0
"	"	"	0.5 cc. of "587" dil. 1:2	4	3	1	0
"	"	"	0.5 cc. of mixture of equal parts of "587" and Serum H/86	4	0	4	0

As is seen from this experiment, a toxic filtrate of a given bacterium consistently neutralized by a homologous serum loses its ability to elicit reactions not only in areas prepared with the filtrate of the same bacterium but also in areas prepared with antigenically unrelated filtrates.

Attempts to Substitute Bacterial Skin Preparatory and Reacting Factors by Various Non-Bacterial Substances

In previous publications (1, 2) a series of experiments was reported to determine whether the local skin reactivity to *B. typhosus* culture filtrate could be produced by skin injections of various non-bacterial substances. In these experiments non-specific irritating substances such as sterile tryptic digest broth and turpentine failed to induce the local skin reactivity. In the experiments to be reported in this paper an additional number of various non-bacterial substances were employed in order to elucidate further the nature of this phenomenon.

Attempts to Induce the Local Skin Reactivity to B. typhosus by Skin Injection of Non-Bacterial Substances

Experiment.—A. The substances used to elicit the skin reactivity were selected with the following purposes in mind: to "block" locally the reticulo-endothelial cells* and to increase the permeability of the capillaries with or without local inflammation. One area of the skin of the abdomen of each rabbit was injected with 0.25 cc. of undiluted "A_{11}" filtrate. Three more skin sites were each injected with 0.25 cc. of various dilutions of the substances tested. A group of four rabbits was used with each substance save India ink which was tested in eight rabbits. The substances tested were as follows:—2, 0.5 and 0.05 per cent of gum tragacanth; 2, 0.5 and 0.05 per cent of crystalline egg albumin; undiluted normal horse serum and normal horse serum previously diluted 1:10 and 1:100; 3, 0.5 and 0.05 per cent of sodium arsenate; 10, 1 and 0.1 per cent of spirits of nitroglycerine and Higgins' waterproof India ink diluted previously 1:2, 1:4, 1:6, 1:20, 1:40, 1:80 and 1:100. The ink was diluted with distilled water and filtered before using. Twenty-four hours after the skin injections each rabbit received a single intravenous injection of 1 cc. per kilo of body weight of "A_{11}" previously diluted 1:20. The reactions were read 4 and 24 hours after the intravenous injections. Not less than three rabbits survived in each group. They all showed severe hemorrhagic necrosis in the areas prepared with "A_{11}" but the *B. typhosus* reacting factors had

* It is not known whether a blockade in the literal sense occurs.

no visible effect upon the skin sites injected with the various non-bacterial substances.

B. Another experiment was done in which rabbits were injected intradermally with sodium arsenate, India ink and normal horse serum in various dilutions as above. Four hours after the rabbits were injected intravenously with "A_{11}" (1 cc. of "A_{11}" diluted 1:20, per kilo of body weight). No skin reactions followed the intravenous injection. Evidently, therefore, shortening the "incubation period" failed to produce local skin reactivity to *B. typhosus*.

As is seen from these experiments, a variety of non-bacterial substances which are able to increase the permeability of capillaries, elicit inflammation and "block" the reticulo-endothelial cells, failed to induce the state of local skin reactivity to *B. typhosus* reacting factors.

Attempts to Reproduce the Phenomenon by Means of Non-Bacterial Substances

Experiment.—A. Six rabbits received each four simultaneous intradermal injections of crystalline egg albumin in various concentrations. The upper right areas of the skin of the abdomen were injected with 0.25 cc. of 10 per cent solution, the lower right areas with 0.25 cc. of 5 per cent solution, the upper left areas with 0.25 cc. of 2 per cent solution and the lower left areas with 0.25 cc. of 1 per cent solution. Twenty-four hours later each of the rabbits received a single intravenous injection of 1 cc. of 10 per cent solution of crystalline egg albumin per kilo of body weight. No reactions followed the intravenous injections.

B. This experiment was similar, except that normal horse serum instead of crystalline egg albumin was used both for skin and intravenous injections. The skin sites of each rabbit were prepared by injection of 0.25 cc. of the serum diluted 1:2, 1:8, 1:16, and 1:32, respectively. Three cc. of undiluted serum per kilo of body weight was injected intravenously 24 hours later. No skin reactions were obtained after the intravenous injections.

As is seen from these experiments non-bacterial protein substances, *i.e.*, normal horse serum and crystalline egg albumin, failed to reproduce the phenomenon of local skin reactivity.

Attempts to Transfer Passively the Local Skin Reactivity to B. Typhosus Culture Filtrates

In these experiments attempts to transfer passively the local skin reactivity were made by using various batches of homologous immune sera derived from various animals (goats, horses and rabbits). Undiluted immune *B. typhosus* sera, and various dilutions of these sera

(1:10, 1:100 and 1:1000) were injected intradermally. Single intravenous injections of potent *B. typhosus* culture filtrates were given either 2, 5, or 24 hours after the skin injections. Since no skin reactions followed the intravenous injections, it was concluded that passive transfer of the local skin reactivity of this phenomenon cannot be realized by the employment of immune sera.

Various Conditions Influencing the Potency of Bacterial Culture Filtrates

This part of the paper embodies a summary of results of a large number of experiments with various culture filtrates. Some of the results have been previously reported.

The skin preparatory and reacting factors fluctuate in potency in the same filtrate. On several occasions there was observed a gradual decrease in the potency as well as complete inactivation of a filtrate stored in the refrigerator for several months. One batch of meningococcus culture filtrate was tested at frequent intervals for a period of 4 months (10). The potency of the filtrate became somewhat lower 4 weeks after its preparation, remained unchanged for 3 months thereafter and suddenly lost its potency altogether 4 months after its preparation. It appears that the meningococcus culture filtrates are more likely to lose their potency than the *B. typhosus* filtrates, although similar difficulties were occasionally experienced with the latter.

On the other hand, some filtrates retain their potency unchanged for a considerable length of time. A filtrate of *B. Shiga* retested more than 14 months after its preparation retained its original strength.

The mode of preparation influences markedly the potency of filtrates, especially of some bacteria. As demonstrated before (6, 7), filtered washings of 24-hour old cultures of *B. typhosus* and meningococcus on solid media* were of greater potency than any fluid culture filtrates thus far obtained. In the case of meningococcus, considerable difficulties are encountered if one attempts to obtain these factors in fluid media. Under apparently the same conditions of preparation, namely,

* Similarly prepared *B. typhosus* filtrates were used by Ferry and Fischer for preparation of agglutinating sera and shown by them to be of superior antigenicity when compared with fluid cultures of the same bacterium (11).

with the same strain of meningococcus, with the same batch of medium of the same pH and the same length of incubation, several cultures made in the course of 1 to 2 weeks may yield filtrates of varying potency and sometimes totally inactive filtrates as well.

The abundance of growth alone is not necessarily an indication of the potency of the filtrates. This observation was made with meningococcus. Enriching substances, such as ascitic fluid, blood and glucose may be inhibitory to the production of the necessary factors. Sometimes cultures very rich in growth were found impotent, while some batches of cultures in plain broth with a comparatively poor growth were quite active.

The meningococcus factors difficult to obtain in fluid cultures are of high potency in washings of young cultures on solid media. *B. typhosus* factors can also be best obtained under these conditions. A *B. typhosus* culture filtrate (on hand at the present moment) prepared by the "agar washings" method is at least fifty times stronger than any fluid culture filtrate of the same strain thus far obtained. It is apparent, therefore, that the production of the skin preparatory and reacting factors is independent of bacterial cell autolysis (6, 7).

Different strains of the same microörganism may vary in their ability to produce these factors.

It was frequently observed that the ability of a given filtrate to kill rabbits was parallel to its skin preparatory and reacting potency. Totally inactive filtrates had no lethal effect even in intravenous doses as large as 3 cc. per kilo of body weight (meningococcus). Very potent "agar washings" filtrates of meningococcus and *B. typhosus*, on the other hand, were able to kill a small percentage of rabbits with as little as 0.005 cc. per kilo of body weight, when injected intravenously. A sudden loss in potency of such a filtrate was also accompanied by a total loss of the lethal effect.

Stock strains of certain microörganisms where filtrates were capable of consistently eliciting the phenomenon (some strains of streptococcus and pneumococcus), after having been kept in the laboratory collection for a considerable length of time, lost their power of producing the skin preparatory and reacting factors. Repeated attempts with these strains yielded only occasionally potent filtrates.

Attempts to Reproduce the Phenomenon of Local Reactivity to Bacterial Culture Filtrates in Various Organs

It seems to be in place to mention here that some preliminary experiments showed the possibility of reproducing this phenomenon in organs other than the skin. Rabbits were used for these experiments. The preparatory injections of bacterial filtrates were made into the renal arteries and intratracheally, respectively, in different rabbits. Twenty-four hours later the filtrates were injected intravenously. Definite lesions resulted in previously prepared organs (*i.e.*, kidney and lungs). This work will constitute the subject of a separate report.

DISCUSSION

The work reported in this paper was done in order to analyze the essential mechanism of local skin reactivity.

The first question which comes up is whether the phenomenon is a manifestation of anaphylaxis. It might be assumed that the skin injection of an antigen brings about local production of antibodies, the area becoming sensitized to a second injection of antigen which enters into combination with the antibodies present in the sensitized area.

There are several important facts which, together, sharply differentiate the phenomenon from the known phenomena of local anaphylaxis. Moreover, as will be seen later, the new data summarized and brought out by the present paper make altogether untenable the hypothesis of the anaphylactic nature of this phenomenon.

The points differentiating the phenomenon were emphasized in previous publications. In brief they are as follows: 1.—The incubation period is of extremely short duration. 2.—The reactivity disappears completely in 48 hours. 3.—The reactivity is induced by a single injection. 4.—The reaction is severe and appears rapidly (1 hour after the intravenous injections it is already possible to distinguish with the naked eye a well defined hemorrhagic necrosis). 5.—The second injection must be made by the intravenous route. All attempts to reproduce the phenomenon by repeated skin injections have failed thus far.* 6.—The phe-

* It is also possible with varying degrees of success to elicit reactions in previously prepared skin sites when the second injection is given intraperitoneally (12).

nomenon cannot be reproduced by non-bacterial substances (*i.e.*, egg albumin, horse serum). 7.—The factors responsible for the phenomenon are only found in certain bacterial cultures and they vary in potency. 8.—The factors are not products of autolysis since conditions of preparation under which very little cell destruction can be expected yield highly potent filtrates. 9.—The skin preparatory and reacting factors are neutralized specifically by immune sera.

These data when compared with well known features of bacterial anaphylaxis and Arthus phenomenon put this phenomenon into an entirely new category.

Once the differentiation is made, it is also possible to show that the essential mechanism of the phenomenon does not depend on the sensitization in the presently accepted view of "anaphylaxis." In fact, if the local preparation consisted of a local production of antibodies, it would be reasonable to expect that the phenomenon would be elicited only with antigens with which these antibodies are able to enter into combination. But, as shown in the present paper, this is not the case. For, on the one hand, the skin preparatory factors of one bacterium prepare the tissue for the factors of another bacterium, even though unrelated. On the other hand, the antibodies against preparatory factors of one bacterium are not able to enter into combination with the reacting factors of another, as demonstrated by the antigenic specificity of the factors derived from various bacteria.

In view of all these considerations the phenomenon seems to be best explained on the basis of a different hypothesis. This can be formulated as follows:

The preparatory factors are endowed with the power of inducing a state of susceptibility or vulnerability in a given area of tissue (*i.e.*, skin and apparently other organs) to substances which have *primary toxicity* but which are not able to attack the tissue under the conditions of natural resistance. The skin preparatory effect is not in the nature of mere trauma, is not due to the local blockade of reticulo-endothelial cells, is not due merely to increased permeability of the capillaries or to inflammation. The vulnerability is probably due to some functional disturbance in the cells which requires a short incubation period for its appearance and which disappears rapidly. For some unknown reason the cells in this state are susceptible to severe and rapidly progressing damage provided the toxic factors are present in the general circulation.

The preparatory factors determining this state as well as the reacting factors responsible for the injury in the susceptible tissue have special characteristic features, as follows:

Both sorts of factors can be accurately titrated; they are filterable and are derived from certain bacterial cultures only; they may partially lose their potency or disappear altogether from a filtrate once highly potent; they are best obtained from young cultures under conditions of insignificant bacterial cell autolysis; their potency does not depend only on the amount of bacterial cells present in a culture; it varies with different strains of the same microörganism and a strain may cease to yield potent factors.

The potency of the factors in a given filtrate seems to parallel its lethal effect upon rabbits.

Hydrogen ion concentrations in the range from 9.0 to 4.0 have no effect upon them. The *B. typhosus* skin preparatory factors show considerable heat resistance (2).

A most interesting and important fact is that the factors determining the state of vulnerability as well as the injury producing factors can be neutralized specifically by immune sera in multiple proportions. Taken together, the properties of the factors suggest very strongly that they are identical with or closely related to true neutralizable toxins. On this assumption one may suppose the phenomenon of local skin reactivity to be the expression of a state of induced susceptibility to neutralizable toxins from microörganisms the great majority of which have not hitherto been shown to produce such toxins (paratyphoid, typhoid, coli, dysentery group, hermorrhagic septicaemia, meningococcus, a few strains of streptococci and pneumococci*).

The results here reported also show the synergistic effect of toxins derived from various bacteria. It has been demonstrated that the induced susceptibility to a toxin of one bacterium can make a tissue receptive to the injurious effect of a large group of apparently biologically unrelated toxins. It is believed that investigations on the effect of these toxins upon different organs and studies on the synergism of

* This phenomenon was also reproduced recently with culture filtrates of *B. pertussis* by Dr. Louis Gross and with culture filtrates of *B. influenzae* by Dr. I. A. Frisch and the author of this paper.

toxins of apparently unrelated microörganisms may elucidate the underlying mechanism of certain pathological conditions (*i.e.*, focal infections resulting in injury to distant organs, complications of infectious diseases produced by unrelated factors, etc.).

CONCLUSIONS AND SUMMARY

The specificity and the nature of the phenomenon of local skin reactivity to various microörganisms have been studied. It has been shown that the skin preparatory and reacting factors of various biologically and serologically unrelated microörganisms are able to substitute for each other, provided they have the power of eliciting the phenomenon for themselves.

Additional evidence has been brought concerning the antigenic specificity of the factors eliciting the phenomenon. A variety of non-bacterial substances which are able to increase the permeability of capillaries, elicit inflammation and "block" the reticulo-endothelial cells, failed to induce the state of local skin reactivity to *B. typhosus* culture filtrate. Non-bacterial protein substances (crystalline egg albumin and normal horse serum) failed to reproduce the phenomenon.

It was not possible to obtain passive transfer of the local skin reactivity.

Various conditions influencing the potency of the bacterial culture filtrates have been pointed out.

The essential nature of the phenomenon has been discussed together with its significance in relationship to disease.

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ORAL IMMUNIZATION AGAINST THE PNEUMOCOCCUS

Use of Bile Salt Dissolved Organisms, etc., Time of Appearance of Immunity and Dosage

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In previous communications (1, 2, 3) it has been shown that white rats can be protected against multiple fatal doses of virulent pneumocci by feeding them (a) tissues of animals killed by the pneumococcus, (b) living pneumococci, and (c) acid killed cells.

The administration of the centrifuged acid killed organisms, either suspended in milk, or desiccated, was no less effective than the feeding of the infected tissue or the living bacteria, conferring on the animals a resistance to 1000 to 10,000 fatal doses. When the cells were centrifuged and heated at 80° for 2 hours, the antigenic property was considerably destroyed. The experiments seemed to show that the ingestion of the growth from 50 cc. per day per rat for a period of 10 to 15 days gave more uniform protection than smaller quantities fed over the same length of time. The duration of the active immunity to Type 1, obtained as a result of feeding for 13 days the tissues of animals killed by the same organism, was at least 4 months, at the end of which time it seemed to be as high as directly after the feeding. An insufficient number of animals prevented tests being made beyond this point. Examination of the sera of orally immunized animals (4) failed to demonstrate the presence of agglutinins or precipitins. Protective antibodies appeared in the sera of animals fed either the infected tissue or the acid killed organisms, seemingly in greater degree in the the former. They could not, however, be detected in the sera of all of the latter examined, and when found their concentration was comparatively low.

The experiments which are being reported here were done to study the effect of feeding (a) the pneumococcus grown in milk and killed by heat, (b) the degraded avirulent pneumococcus (c) the mechanic-

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ally disrupted cell, (d) the bile salt dissolved cell in varying amounts. There are also recorded the results of experiments in which were studied (e) the number of feedings required to produce an immunity, (f) the time at which protection first appears, (g) whether treated animals can resist subcutaneous as well as intraperitoneal injection, and (h) the effect of feeding much smaller quantities of acid killed pneumococci than have been used heretofore. All the experiments were done with Type 1, using white rats, and injecting the test dose in a volume of 0.20 cc. intraperitoneally, except in measuring subcutaneous resistance in (g) above. Except in the experiment in which desiccated material was employed, the organisms were grown daily.

The attempt has been made in the present work to find the form of material, which when administered by mouth, would yield the greatest degree of protection most promptly with the minimum number of feedings and the minimum size of the dose. No emphasis has been placed on the duration of the immunity although figures are given in the tables which show the intervals between the last feeding and the days of the test. We are trying now to learn what determines the length of time during which the animal possesses the increased resistance. It seems at present that the number of feedings plays a part.

Feeding the Pneumococcus Grown in Sterile Milk and Killed by Heat.—

The sterile milk was inoculated with broth cultures and incubated at 37° for about 20 hours. The culture was killed by heating in a water bath for 1 hour at 60°C. It was then placed in the cage with the other food.

Two experiments were done. The results of the first are given in Table I and show that the pneumococcus, when fed in this form, in amounts equivalent to 3 cc. per rat per day, will produce an increased resistance to the virulent organism.

The extent of the protection is not so great as was obtained when larger quantities of the acid killed organisms were fed, either freshly centrifuged and resuspended in milk, or desiccated and mixed with moistened cracker meal. Accurate comparison with the effect of feeding the growth from equal volumes of broth grown organisms obtained in our earlier experiments, is somewhat difficult to make, because in those experiments the treated animals were tested with excessively large doses, and most of them succumbed. Later in the present paper additional data are given with regard to the ingestion of acid killed bacteria from 1 cc. and 5 cc. growth in broth.

The number of days on which the bacteria were fed varied between 11 and 17, but this caused no detectable difference in the degree of protection produced. The rats used in a second experiment were considerably larger than those comprising Table I and gave less uniform results. This difference in response to the same treatment is probably not owing to a difference in the age of the animals since it was not observed where acid killed pneumococci were fed (3). Heating the cultured milk at 60°C. for 1 hour apparently did not destroy the constituent of the cell responsible for the production of the immunity.

TABLE I

Immunizing Effect of Feeding Heat Killed Pneumococcus Milk Culture

C = Control; E = Treated rat; D = Died, days; S = Survived

Wt.	Dose	Result	No. of days fed	°Interval	Wt.	Dose	Result	No. of days fed	°Interval	Wt.	Dose	Result	No. of days fed	°Interval
gm.	*cc.*				*gm.*	*cc.*				*gm.*	*cc.*			
*C158	10^{-7}	D2			E154	10^{-6}	D3	15	1	E273	10^{-6}	D2	14	21
C165	10^{-6}	D2			E171	10^{-5}	S	15	1					
E158	10^{-7}	S	15	1						*C213	10^{-8}	S		
E160	10^{-6}	S	15	1	*C168	10^{-8}	S			C225	10^{-7}	D3		
					C170	10^{-7}	D4			C227	10^{-6}	D2		
*C138	10^{-8}	D2			C176	10^{-6}	D2			E195	10^{-6}	S	11	22
C148	10^{-7}	D2			E144	10^{-6}	S	16	1	E203	10^{-5}	S	11	22
C149	10^{-6}	D3			E185	10^{-5}	S	16	1	E213	10^{-5}	D2	11	22
E134	10^{-7}	S	17	1	E188	10^{-5}	S	16	1					
E144	10^{-6}	S	17	1						*C233	10^{-7}	D2		
					*C206	10^{-8}	D2			C241	10^{-6}	D3		
*C155	10^{-8}	D3			C229	10^{-7}	D2			E194	10^{-6}	D3	11	28
C161	10^{-7}	D3			C237	10^{-6}	D2			E214	10^{-5}	D2	11	28
C169	10^{-6}	D3			E191	10^{-6}	D3	11	21	E227	10^{-5}	S	11	28
E145	10^{-7}	S	15	1	E213	10^{-5}	D2	11	21					

* A new day.

The average amount ingested was 3 cc. per day for the number of days indicated.

° Elapsed number of days between last feeding and test.

Feeding the Degraded Avirulent Pneumococcus.*—This strain of Type 1 pneumococcus was derived from the virulent strain used in the

* In a preliminary communication (*Proc. Soc. Exp. Biol. and Med.*, 1929, 26, 565–567) this organism was referred to as "avirulent." It would seem more accurate to describe it as "degraded avirulent" in view of its properties.

other parts of this work by adding 1 volume of "N" HCl acid solution to 14 volumes of glucose meat extract broth culture, and subculturing before the growth was killed.

The organism failed to kill mice when 1 cc. of a 24 hour culture was injected intraperitoneally. It appeared at first to be entirely insoluble in sodium glyco-

TABLE II

Effect of Feeding HCl Killed Degraded Avirulent Pneumococcus

Wt.	Dose	Result	°Interval	Wt.	Dose	Result	°Interval	Wt.	Dose	Result	°Interval
gm.	*cc.*			*gm.*	*cc.*			*gm.*	*cc.*		
*C70	10^{-8}	D2		E116	10^{-8}	D3	1	C104	10^{-7}	D2	
C74	10^{-7}	D1		E120	10^{-7}	D2	1	C110	10^{-6}	D2	
C77	10^{-6}	D2		E144	10^{-6}	D4	1	E93	10^{-9}	S	5
E61	10^{-8}	D2	12					C108	10^{-8}	S	5
E66	10^{-7}	D3	12	*C118	10^{-8}	S		E108	10^{-7}	D3	5
E68	10^{-6}	D2	12	C133	10^{-7}	D3					
				E127	10^{-8}	D2	3	*C102	10^{-8}	D3	
*C138	10^{-8}	D2		E129	10^{-7}	D4	3	C103	10^{-7}	D2	
C144	10^{-7}	D2		E131	10^{-6}	D2	3	C122	10^{-7}	D2	
C158	10^{-6}	D2						C123	10^{-6}	D2	
E135	10^{-8}	D**	14	*C105	10^{-8}	D5		E122	10^{-8}	S	8
E140	10^{-7}	D2	14	C109	10^{-7}	D2		E123	10^{-8}	S	8
E147	10^{-6}	D2	14	E148	10^{-8}	D2	4	E127	10^{-8}	S	8
				E175	10^{-7}	D2	4	E127	10^{-7}	D4	8
*C118	10^{-8}	D2		E185	10^{-6}	D1	4	E133	10^{-7}	D3	8
C118	10^{-7}	D2						E138	10^{-7}	D3	8
C144	10^{-6}	D2		*C90	10^{-9}	S		E146	10^{-6}	D2	8
				C94	10^{-8}	D2					

* A new day.

** Very sick; killed.

The average amount ingested was equivalent to 50 cc. growth per day, for 3 days in the case of the first 2 groups, and for 9 days in the remaining 5 groups.

° Elapsed number of days between last feeding and test.

cholate, but on adding considerably larger quantities of this salt than sufficed to dissolve the virulent strain, solution resulted. Organisms removed from blood agar and suspended in 0.90 per cent salt solution appear to dissolve somewhat more easily than broth cultures. No capsule could be demonstrated.

It was agglutinated approximately to the same extent when equal volumes (0.5 cc.) of the culture and 1–20 Type 1, 1–20 Type 2, and 1–5 Type 3 antisera were

mixed and incubated at 37°C.* The precipitin test performed by mixing 0.5 cc. of a Berkefeld filtrate of a beef heart culture with 0.5 cc. of 1–10 Type 1, 1–10 Type 2 and 1–5 Type 3 antisera showed no precipitate after incubating for 20 hours at 37°C.

Two experiments were done with this strain. In the first, the organism was grown in glucose meat extract broth, and before use was treated with "N" HCl

TABLE III

Effect of Feeding Degraded Avirulent Pneumococcus

Wt.	Dose	Result	Wt.	Dose	Result
gm.	*cc.*		*gm.*	*cc.*	
*C57	10^{-8}	S	**C60	10^{-8}	S
C61	10^{-8}	S	C61	10^{-8}	S
C63	10^{-8}	D3	C72	10^{-8}	D3
C68	10^{-8}	D2	C80	10^{-8}	D2
C70	10^{-7}	D2	C90	10^{-7}	D2
C79	10^{-7}	D2	C92	10^{-7}	D3
E57	10^{-8}	S	C91	10^{-6}	D2
E60	10^{-8}	S	C95	10^{-6}	D2
E64	10^{-8}	D2	E62	10^{-9}	S
E69	10^{-8}	S	E67	10^{-9}	S
E70	10^{-7}	S	E75	10^{-8}	D3
E79	10^{-7}	D2	E76	10^{-8}	D2
			E75	10^{-7}	D2
			E81	10^{-7}	D3
			E82	10^{-6}	D2
			E91	10^{-6}	D3

* Three days after the last feeding.

** Six days after the last feeding.

The average amount ingested was equivalent to 50 cc. growth per day per rat on each of 3 consecutive days. The culture, although made up to N/15 HCl and kept at room temperature for 2 hours (at which time feeding took place), was still alive.

acid solution to make a "N/15" concentration and was left at room temperature for 2 hours, at which time subcultures demonstrated that the growth had been killed. The quantity fed was equivalent to 50 cc. growth per rat per day. The

* Similar tests done at 56°C. with Types 1 to 13 pneumococcus antisera showed about the same degree of agglutination in all. Type numbers are those used at Bureau of Laboratories, Department of Health, New York City.

first two groups of E rats listed in Table II were fed this amount on 3 successive days. The remaining 5 groups received 6 additional feedings and then the resistance of all the rats was measured. It will be seen that the fatal dose for controls is, with one exception, 10^{-8} cc. The treated rats succumb to the same dose, except on 2 days when 10^{-7} cc. was required to kill. On the day when 10^{-7} cc. was required to kill a control, 10^{-8} cc. was fatal for an E rat. At first, the survival of the E rats injected with 10^{-8} cc. in the last two groups was regarded as the result of chance, especially since in one of the preceding groups a control survived 10^{-8} cc. whereas an E rat succumbed to this dose. It was thought that no protection followed ingestion of this organism.

The experiment recently has been repeated with some modification. The pneumococcus was grown in beef heart broth without glucose, and 3 consecutive daily feedings were given, each equivalent to the growth of 50 cc. per rat. As in the first experiment, HCl acid was added to make N/15 concentration, but subcultures at this time showed that the culture had not been killed. It would seem that this organism was somewhat more resistant to acid than the virulent one. Table III gives the data obtained when the rats were injected with virulent Type 1. Three days after the last feeding one of the treated rats survived one fatal dose, another ten fatal doses. Six days following the last ingestion of bacteria none of the treated rats survived single fatal doses or 10 and 100 fold multiples of this dose. The survival of the other two animals suggests, when considered with the survival of some of the treated rats in Table II, that, though the protection afforded by feeding the degraded Type 1 pneumococcus is slight, it nevertheless seems to exist.

Feeding Mechanically Disrupted Cells.—The object of this experiment was to determine whether the intact cell is essential in this method of immunization or whether the component parts would function equally well.

The organism used was a virulent strain. The bacteria were desiccated in a partial vacuum and pulverized in a pebble mill for 18 hours. Microscopic examination failed to show any whole cells. There were, in all probability some intact organisms in this powder, but it is not likely they were sufficiently numerous to produce the high degree of immunity observed. After distributing the disrupted cells in milk, the whole was placed in the cage. The rats had been fasted 18 hours daily and the volume of milk was kept small, so that all of the mixture was quickly consumed. Feeding took place on 16 days, each rat receiving the equivalent of 50 cc. growth per day. Table IV shows the results. The treated rats survived 1000 and 10,000 fatal doses.

The protection on the whole is as great and as uniform as when the acid killed intact organism is used. It is apparent that it is not neces-

sary to feed the intact pneumococcus; the derivatives alone are sufficient. This result is confirmed by the experiments done with bile salt dissolved bacteria.

Feeding Bile Salt Dissolved Pneumococci.—Several experiments were done with dissolved cells in order to confirm the observation that the whole cell is not indispensable for producing an immunity by feeding. It seemed moreoever that, if it should prove to be effective, the ingestion of the material in solution might yield very uniform results.

TABLE IV

Immunizing Effect of Feeding Desiccated Mechanically Disrupted Pneumococcus

Wt.	Dose	Result	°Interval	Wt.	Dose	Result	°Interval	Wt.	Dose	Result	°Interval
gm.	*cc.*			*gm.*	*cc.*			*gm.*	*cc.*		
*C157	10^{-8}	S		E164	10^{-6}	S	16	E132	10^{-6}	S	19
C166	10^{-7}	D8		E170	10^{-5}	S	16	E133	10^{-5}	S	19
E152	10^{-8}	S	6					E142	10^{-4}	S	19
E168	10^{-7}	S	6	*C174	10^{-8}	S					
				C180	10^{-7}	D2		*C198	10^{-8}	D2	
*C143	10^{-8}	S		C211	10^{-6}	D2		C205	10^{-7}	D2	
C170	10^{-7}	D6		E176	10^{-6}	S	18	C214	10^{-6}	D2	
E138	10^{-7}	S	9	E153	10^{-5}	S	18	E97	10^{-6}	S	21
E138	10^{-6}	S	9	E167	10^{-4}	D3	18	E117	10^{-6}	S	21
								E131	10^{-5}	S	21
*C169	10^{-8}	S		*C173	10^{-8}	S		E138	10^{-5}	S	21
C182	10^{-7}	D2		C179	10^{-7}	D2		E159	10^{-4}	S	21
C187	10^{-6}	D2		C181	10^{-6}	D2		E158	10^{-4}	D3	21
E158	10^{-6}	S	16								

* A new day.

Each rat received the equivalent of 50 cc. growth per day on each of 16 days.

° Elapsed number of days between last feeding and test.

The organism was grown in beef heart broth. The centrifuged bacteria were dissolved in sodium taurocholate. In the first two experiments of this kind, the solution was not filtered. Cracker meal was added, the whole was intimately mixed and placed in the cage. A preliminininary period of fasting was employed to ensure better absorption of the active material. Each rat received the equivalent of 50 cc. growth per day for 21 days. The data of this experiment are recorded in Table V, and show that bile salt dissolved pneumococci, when administered by mouth create a consistent increased resistance against the virulent organism.

The experiment was repeated, both the quantity fed daily and the number of feedings being reduced. The data are given in Table VI. The first group of rats

TABLE V

Immunizing Effect of Feeding Sodium Taurocholate Dissolved Pneumococcus

Wt.	Dose	Result	°Interval	Wt.	Dose	Result	°Interval
gm.	*cc.*			*gm.*	*cc.*		
*C130	10^{-8}	D3		*C132	10^{-8}	D6	
C150	10^{-7}	D8		C134	10^{-7}	D6	
E194	10^{-7}	S	13	C137	10^{-6}	D2	
E199	10^{-7}	S	13	E174	10^{-8}	S	24
				E176	10^{-7}	S	24
*C146	10^{-8}	Sick but recovered		E228	10^{-6}	D2	24
C148	10^{-7}	D5					
E161	10^{-7}	S	14	*C160	10^{-8}	S	
E182	10^{-6}	D3	14	C175	10^{-7}	D2	
				C186	10^{-6}	D4	
*C132	10^{-8}	D6		E225	10^{-8}	S	26
C138	10^{-7}	D6		E227	10^{-7}	S	26
E192	10^{-8}	S	17	E254	10^{-6}	S	26
E198	10^{-7}	S	17				
E199	10^{-6}	S	17	*C151	10^{-8}	D2	
				C158	10^{-7}	D2	
*C135	10^{-8}	D2		C171	10^{-6}	D2	
C138	10^{-7}	D		E232	10^{-8}	S	28
C150	10^{-6}	D6		E233	10^{-7}	S	28
E199	10^{-8}	S	20	E247	10^{-6}	D2	28
E207	10^{-7}	S	20				
E208	10^{-6}	D2	20	*C157	10^{-8}	S	
				C166	10^{-7}	D8	
				**E184	10^{-7}	S	41
				**E200	10^{-7}	S	41

* A new day.

** Had litter 1 month before the test, which was 10 days after last feeding.

The treated rats received 21 feedings, each equivalent to 50 cc. growth per rat. The feedings extended over a period of 5 weeks and ceased 2 weeks before the first group in the above table was tested.

° Elapsed number of days between last feeding and test.

shown in the table was fed the equivalent of the growth from 25 cc. culture per rat on each of 3 consecutive days. Six days after the last feeding they were tested along with controls. The E rat which survived 10^{-6} cc. illustrates that an in-

creased resistance had already been built up. The E rats omprising the second and third groups, in addition to having received the 3 feedings mentioned, were now given 1 and 2 additional feedings respectively. The animals of the remaining 4 groups were fed 6 times in all. Examination of the table shows that the ingestion of the bile salt dissolved organisms was followed by the ability to resist 10,000 fatal doses of the organism.

TABLE VI

Immunizing Effect of Feeding Sodium Taurocholate Dissolved Pneumococcus

Wt.	Dose	Result	°Interval	Wt.	Dose	Result	°Interval	Wt.	Dose	Result	°Interval
gm.	cc.			gm.	cc.			gm.	cc.		
*C53	10^{-8}	S		E81	10^{-7}	S	1	E91	10^{-4}	S	3
C60	10^{-7}	D2		E83	10^{-6}	S	1				
C63	10^{-6}	D1		E84	10^{-5}	S	1	*C77	10^{-8}	D3	
E52	10^{-8}	S	6					C82	10^{-7}	D2	
E59	10^{-7}	D2**	6	*C70	10^{-8}	D3		C91	10^{-6}	D2	
E66	10^{-6}	S	6	C74	10^{-7}	D5		E106	10^{-7}	S	7
				C81	10^{-6}	D2		E111	10^{-6}	S	7
*C70	10^{-8}	D2		E71	10^{-7}	S	1	E115	10^{-5}	S	7
C74	10^{-7}	D1		E77	10^{-6}	S	1	E122	10^{-4}	S	7
C75	10^{-6}	D1		E82	10^{-5}	S	1				
E72	10^{-8}	S	3					*C90	10^{-8}	D2	
E73	10^{-7}	S	3	*C68	10^{-8}	D2		C94	10^{-7}	D2	
E80	10^{-6}	S	3	C77	10^{-7}	D2		C102	10^{-6}	D2	
				C79	10^{-6}	D1		†E89	10^{-6}	D4	10
*C72	10^{-8}	S		E82	10^{-7}	S	3	E100	10^{-5}	S	10
C75	10^{-7}	D2		E86	10^{-6}	S	3	E128	10^{-4}	S	10
C76	10^{-6}	D2		E91	10^{-6}	S	3	E158	10^{-3}	D1	10

* A new day.

** Ill before injection.

† Has an infected leg.

The E rats in the first group received 3 feedings, those in the second and third groups 4 and 5 feedings respectively, and the remaining ones 6 feedings, each equivalent to 25 cc. growth. In addition each E rat was fed 2.5 mg. sodium glycocholate.

° Elapsed number of days between last feeding and test.

In the two experiments just described, there remained the possibility that the intact cells, though few in number, might have been responsible for the immunity which was created. Hence in all the subsequent experiments, the bile dissolved material was passed through a Berkefeld filter before it was fed to the rats.

It now appeared that a portion, at least, of the increased resistance could be elicited by 3 consecutive feedings. It was then decided to determine whether 3 consecutive feedings would be followed by the maximum degree of protection, and to make the first test of the animals' resistance 24 hours after the last of 3 such feedings instead of 6 days as was the case with those rats in the preceding experiment which were fed only 3 times.

TABLE VII

Immunizing Effect of Feeding Berkefeld Filtrate of Sodium Glycocholate Dissolved Pneumococcus

Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
gm.	*cc.*		*gm.*	*cc.*		*gm.*	*cc.*	
*C62	10^{-8}	S	*C71	10^{-8}	D3	*C82	10^{-8}	D2
C83	10^{-7}	D2	C72	10^{-7}	D2	C84	10^{-7}	D2
E63	10^{-8}	S	E55	10^{-7}	S	C105	10^{-6}	D2
E83	10^{-7}	S	E65	10^{-6}	D2	**E72	10^{-7}	D2
			E71	10^{-5}	D3	**E73	10^{-6}	D2
*C63	10^{-8}	D2				**E73	10^{-6}	S
C85	10^{-7}	D2	*C84	10^{-8}	S	**E80	10^{-5}	S
E62	10^{-7}	S	C87	10^{-7}	D2	**E114	10^{-5}	S
E87	10^{-6}	S	C103	10^{-6}	D2			
			E61	10^{-7}	D2	*C90	10^{-8}	D3
*C51	10^{-9}	S	E64	10^{-7}	S	C93	10^{-7}	D3
C70	10^{-8}	D2	E68	10^{-6}	S	C95	10^{-6}	D2
C72	10^{-7}	D2	E69	10^{-6}	S	**E60	10^{-6}	D2
E51	10^{-7}	S	E75	10^{-5}	S	**E66	10^{-6}	D4
E71	10^{-6}	S	E116	10^{-5}	S	**E66	10^{-5}	S
E73	10^{-5}	S				**E79	10^{-5}	D2

* A new day.

** These rats ate of the cracker meal-filtrate organism mixture indifferently and it is not certain that some of them ate any. Feeding of filtrate took place on the 3 days immediately preceding the day on which the first group above was injected. The remaining 6 groups were injected 4, 5, 7, 8, 10 and 12 days, respectively, after the last feeding. Average amount fed was equivalent to 30 cc. culture per rat each time, together with 5 mg. sodium glycocholate.

Each rat was fed the Berkefeld filtrate of sodium glycocholate dissolved pneumococci equivalent to 30 cc. culture per day on 3 successive days. The rats were fasted for 18 hours and the cracker meal-filtrate mixture was placed in the cage. Unfortunately some of the animals ate of it indifferently and one could not be

certain that every rat had eaten at least a portion. A rough separation into 2 groups was made, comprising those animals which had eaten liberally and those observed to have barely taken any. The results of the test appear in Table VII, and show that 3 feedings of cell free filtrate protected rats against 1000 fatal doses. At least a portion of this immunity appeared 24 hours after the last dose; all of it was present 5 days after the last dose. The irregularity in the reaction of the rats comprising the last 2 of the 7 groups is probably owing to the fact that these animals were the ones which were observed to have eaten sparingly of the mixture of filtrate and cracker meal.

TABLE VIII

Time of Appearance of Immunity Produced in Rats by Feeding a Single Dose of Sodium Glycocholate Dissolved Pneumococcus (Berkefeld Filtrate)

Tested 24 hrs. after the feeding			Tested 48 hrs. after the feeding		
Wt.	Dose	Result	Wt.	Dose	Result
gm.	*cc.*		*gm.*	*cc.*	
C74	10^{-8}	D2	C64	10^{-8}	D2
C65	10^{-8}	D3	C71	10^{-8}	D2
C79	10^{-7}	D2	C81	10^{-7}	D2
C82	10^{-7}	D2	C82	10^{-7}	D2
C85	10^{-6}	D2	C82	10^{-6}	D2
C92	10^{-6}	D2	C90	10^{-6}	D2
E71	10^{-8}	S	E70	10^{-8}	S
E78	10^{-8}	D3	E71	10^{-8}	S
E80	10^{-7}	S	E73	10^{-7}	S
E80	10^{-7}	D3	E80	10^{-7}	S
E82	10^{-6}	D2	E91	10^{-6}	S
E114	10^{-5}	D2	E93	10^{-5}	S

Each E rat received the equivalent of 50 cc. growth and 8 mg. sodium glycocholate.

This experiment definitely indicated that the presence of the whole cell was not essential in the production of an immunity by the oral route.

It was now known that animals fed the sterile filtrate on 3 consecutive days were at least partially protected 72 hours after the first feeding. This suggested the advisability of determining more exactly the degree of protection at such early stages, and of learning whether the immunity might appear sooner than 72 hours after the first ingestion, and possibly after fewer than 3 feedings.

In the next experiment (Table VIII) each rat was fed the Berkefeld filtrate of sodium glycocholate dissolved pneumococci equivalent to 50 cc. growth in a single feeding. Each rat was fed separately, to be certain that every animal received its portion. One half of the treated rats were tested 24 hours later and the other half 48 hours after the feeding. In the 24 hour test one rat survived a single fatal dose and one survived 10 such doses. Two other E rats receiving the same amounts and the remaining 4 injected with larger quantities, succumbed. Forty-eight hours after the ingestion of the bile dissolved bacteria however, all 6 treated rats, injected with quantities varying from 1 to 1000 fatal doses survived. This

TABLE IX

Time of Appearance of Immunity Produced by Feeding a Single Dose of Sodium Glycocholate Dissolved Pneumococcus (Berkefeld Filtrate)

Tested 24 hrs. after the feeding			Tested 48 hrs. after the feeding		
Wt.	Dose	Result	Wt.	Dose	Result
gm.	*cc.*		*gm.*	*cc.*	
C60	10^{-8}	D3	C70	10^{-8}	D2
C75	10^{-8}	D3	C80	10^{-8}	S
C75	10^{-7}	D2	C87	10^{-7}	D2
C78	10^{-7}	D2	C93	10^{-7}	D2
C100	10^{-6}	D2	C97	10^{-6}	D2
E85	10^{-8}	D3	E90	10^{-8}	S
E85	10^{-8}	D2	E99	10^{-8}	S
E87	10^{-7}	D2	E95	10^{-7}	S
E95	10^{-7}	D2	E93	10^{-7}	S
E95	10^{-6}	D3	E72	10^{-6}	S
E95	10^{-5}	D2	E112	10^{-5}	S

Each rat received the equivalent of 50 cc. growth and 5 mg. sodium glycocholate.

experiment was repeated, in all its details, except that the quantity of bile salt used to dissolve the cells was reduced so that each rat was given 5 mg. instead of 8. Subsequently this was even further reduced. The data appear in Table IX. This time none of the E rats survived when tested 24 hours after the single ingestion. All those injected after 48 hours survived.

It seems therefore that the increased resistance is present fairly consistently and rather marked at the end of 48 hours.

Having found that the immunity is created so promptly it was next decided to learn whether the quantity of culture fed could be reduced

from 50 to 5 cc. without delaying the time of its appearance or reducing its extent.

Table X shows that one treated rat survived an injection of 10 fatal doses 24 hours following a single feeding equivalent to 5 cc. of growth. At the end of 47 hours the treated rats were able to tolerate at least 1000 fatal doses and after 72 hours, 10,000 such doses.

It seemed worth while to study the effect of feeding still smaller amounts, and to determine whether the treated rats would survive

TABLE X

Time of Appearance of Immunity by Feeding a Single Dose of Sodium Glycocholate Dissolved Pneumococcus (Berkefeld Filtrate)

24 hrs. after feeding			47 hrs. after feeding			72 hrs. after feeding		
Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
gm.	*cc.*		*gm.*	*cc.*		*gm.*	*cc.*	
C74	10^{-8}	D2	C91	10^{-8}	D4	C106	10^{-8}	D3
C75	10^{-8}	D2	C96	10^{-8}	D4	C116	10^{-7}	D2
C79	10^{-7}	D2	C101	10^{-7}	D3	E76	10^{-7}	S
C84	10^{-7}	D2	C107	10^{-7}	D3	E86	10^{-6}	S
E70	10^{-8}	D2	E86	10^{-8}	S	E98	10^{-5}	S
E78	10^{-8}	D2	E86	10^{-8}	S	E107	10^{-4}	S
E83	10^{-7}	D2	E86	10^{-7}	S			
E84	10^{-7}	S	E92	10^{-7}	S			
			E93	10^{-6}	S			
			E94	10^{-5}	S			

Each rat received the equivalent of 5 cc. growth and 1 mg. sodium glycocholate.

subcutaneous injection of the pneumococci as well as intraperitoneal. The experiments which were done to find the answer to the latter will be described first.

A group of rats was fed the Berkefeld filtrate of sodium glycocholate dissolved pneumococci. Twenty-four hours later (Table XI) two of this group were injected subcutaneously with 10^{-8} cc., and two with 10^{-7} cc. Four controls were similarly injected. There was no indication of the presence of any immunity at this time. Forty-eight hours following the feeding, several additional animals were injected and the result was the same. After 72 hours the remaining rats of the group were tested. The fatal dose for the controls was between 10^{-8} and 10^{-7} cc. Among

the treated rats, the one injected with 10^{-5} cc. died, those receiving smaller doses and the rat receiving 10^{-4} cc. survived. The results of this experiment seemed to show that the resistance to a subcutaneous injection appears 1 day later than to an intraperitoneal one. The survival of the animal injected with 10^{-4} cc. indicates that the degree of protection may be as high when tested by the subcutaneous route as by the intraperitoneal. A repetition of this experiment is illustrated in Table XII. Forty-eight hours after the feeding four treated rats were injected with 10^{-8}, 10^{-7}, 10^{-7} and 10^{-6} cc., respectively; of these, one (10^{-8} cc.) died, and the heart's blood was found to be sterile. Of four untreated rats injected subcu-

TABLE XI

Immunity to Subcutaneous Injection Produced by Feeding a Single Dose of Sodium Glycocholate Dissolved Pneumococcus (Berkefeld Filtrate)

24 hrs. after feeding			48 hrs. after feeding			72 hrs. after feeding		
Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
gm.	*cc.*		*gm.*	*cc.*		*gm*	*cc.*	
C77	10^{-8}	S	C75	10^{-8}	S	C81	10^{-8}	D5
C81	10^{-8}	S	C79	10^{-8}	S	C80	10^{-8}	S
C83	10^{-7}	S	C86	10^{-7}	S	C62	10^{-7}	D3
C88	10^{-7}	D4	C80	10^{-7}	D3	C83	10^{-7}	D3
E77	10^{-8}	S	E71	10^{-8}	S	C93	10^{-7}	D3
E80	10^{-8}	D5	E76	10^{-8}	S	C101	10^{-}	D5
E84	10^{-7}	D4	E76	10^{-7}	S	E70	10^{-}	S
E88	10^{-7}	S	E76	10^{-7}	D8	E77	10^{-7}	S
			E89	10^{-6}	D3	E88	10^{-6}	S
			E91	10^{-5}	D3	E92	10^{-5}	D3
						E106	10^{-4}	S

Each rat received the equivalent of 5 cc. growth and 1 mg. sodium glycocholate.

taneously with 10^{-8}, 10^{-7}, 10^{-7} and 10^{-6} cc., respectively, only one (10^{-7} cc.) lived. Seventy-two hours after the single feeding 10^{-6} cc. was fatal for each of two controls. Among the E rats, only one (10^{-8} cc.) died. The other rat injected with this dose, as well as two injected with 10^{-7} cc., two with 10^{-6} cc., three with 10^{-5} cc. and two with 10^{-4} cc. all survived.

Judging by these data it would seem as if the resistance to subcutaneous injection may also appear 48 hours after a single feeding. The remaining E rats of this group were injected intraperitoneally 96 hours after ingestion of the filtrate and survived 1, 10, 100 and 1000 fatal doses; one of two injected with 10,000 such doses also lived.

An effort was next made to measure simultaneously the immunizing value of feeding 0.1, 1, 5, and 50 cc. growth, in the form of Berkefeld filtrate of sodium glycocholate dissolved pneumococci, and of 50 cc. growth in the form of acid killed sedimented whole cells. The primary

TABLE XII

Immunity to Subcutaneous and Intraperitoneal Injection Produced by Feeding a Single Dose of Sodium Glycocholate Dissolved Pneumococcus (Berkefeld Filtrate)

Subcutaneous injection						Intraperitoneal injection		
48 hrs. after feeding			72 hrs. after feeding			96 hrs. after feeding		
Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
gm.	*cc.*		*gm.*	*cc.*		*gm.*	*cc.*	
C52	10^{-8}	D16	C59	10^{-8}	S	C67	10^{-8}	S
C56	10^{-7}	D12	C65	10^{-8}	S	C71	10^{-8}	S
C62	10^{-7}	S	C73	10^{-7}	S	C76	10^{-7}	D3
C64	10^{-6}	D3	C73	10^{-7}	S	C80	10^{-7}	D2
*E52	10^{-8}	D7	C75	10^{-6}	D15	C86	10^{-6}	D2
E55	10^{-7}	S	C76	10^{-6}	D3	C90	10^{-6}	D2
E60	10^{-7}	S	C78	10^{-5}	D2	C94	10^{-5}	D2
E68	10^{-6}	S	E54	10^{-8}	D16	E68	10^{-7}	S
			E63	10^{-8}	S	E74	10^{-6}	S
			E65	10^{-7}	S	E75	10^{-6}	S
			E66	10^{-7}	S	E75	10^{-5}	S
			E66	10^{-6}	S	E77	10^{-5}	S
			E68	10^{-6}	S	E82	10^{-4}	S
			E72	10^{-5}	S	E84	10^{-4}	S
			E73	10^{-5}	S	E84	10^{-3}	D2
			E73	10^{-5}	S	E89	10^{-3}	S
			E75	10^{-4}	S	E90	10^{-2}	D2
			E76	10^{-4}	S	E98	10^{-2}	D2

* Heart's blood sterile. None of the others was examined.

Each rat received the equivalent of 5 cc. growth and 1 mg. of sodium glycocholate.

purpose was to learn whether feeding the equivalent of less than 5 cc. culture (bile salt dissolved) would prove as effective as feeding the equivalent of 5 cc. or more.

Feeding the acid killed bacteria from 50 cc. culture and the bile salt dissolved bacteria from the same quantity was included in order to learn whether there would

be any difference in effect produced by using what now appeared to be a much larger single dose of the latter than is necessary, and an equally large single dose of the former. The quantity fed (growth from 50 cc.) being definitely known to be more than sufficient, a poorer result with the acid killed bacteria could be interpreted as indicating an advantage in the use of a solution, due either to the ease with which such material can be absorbed or to the presence of the small amount of bile salt. By feeding all five groups at the same time, a more accurate comparison was expected than by dividing up the experiment. The organisms were grown in beef heart broth. Each of the several quantities required for the other groups of rats was centrifuged separately in order to make the dosage as accurate as possible. Since the quantity required for the animals receiving 0.1 cc. each was small, four times this amount was centrifuged and dissolved in four times the weight of sodium glycocholate needed, and after the material was filtered one-fourth of it was measured out, mixed with cracker meal and fed to each rat separately, just as for the remaining animals. The weight of bile salt ingested by each rat was 1 mg., except in the case of the animals fed the bile dissolved growth from 50 cc.; on account of the larger quantity of organisms, 4 mg. of sodium glycocholate were used. The animals were fasted for 18 hours before ingestion of the bacteria and for about 4 hours after. Twenty-four hours following the feeding of the filtrates the treated rats and controls were injected intraperitoneally with graded doses of pneumococci. The entire feeding part of the experiment was repeated twice more with new rats in order to measure the immunity after 48 and 72 hours. Table XIII gives the results. In the 24 hour test the amounts injected varied from 10^{-8} to 10^{-5} cc. in each of the six groups including the controls. There were no survivals either among the controls or those fed 0.1 cc. Among the animals in the 1 cc. group, two survived, one after an injection of 10^{-8} cc., the other following 10^{-7} cc. In the 5 cc. group, one survived 10^{-6} cc., and in the 50 cc. group one rat which was injected with 10^{-8} cc. lived. None survived among those fed the acid killed bacteria.

These results confirm the fact already observed that the bile salt dissolved bacteria from 50 cc. will produce neither an appreciably larger nor a more rapid response than the organisms from 5 cc.

The 48 hour test is difficult to interpret because of the apparent irregularity in the results.

The customary effect of feeding either 5 or 50 cc. is absent. The comparatively poor results may be due to the fact that just before the feeding all the animals were kept in a very small cage with only a small opening for air. The weather was very hot and humid and when the animals were taken out they were quite weak and prostrated and one had died. The 48 hour test with the 0.1 cc. and 1 cc. quantities, offers no aid in determining to what extent the equivalent of less than 5 cc. growth will prove of value in producing an immunity in this period of time. The

TABLE XIII

Comparative Immunizing Values of a Single Feeding of Varying Quantities of Pneumococcus. (Berkefeld Filtrate)

Test done 24 hrs. after feeding the filtrate			Test done 48 hrs. after feeding the filtrate			Test done 72 hrs. after feeding the filtrate		
Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
				Controls				
gm.	*cc.*		*gm.*	*cc.*		*gm.*	*cc.*	
58	10^{-8}	D2	49	10^{-8}	D4	67	10^{-9}	S
66	10^{-8}	D3	57	10^{-8}	D3	74	10^{-9}	S
78	10^{-7}	D3	62	10^{-7}	D3	82	10^{-8}	D2
73	10^{-7}	D3	68	10^{-7}	D4	100	10^{-8}	D2
70	10^{-6}	D2	74	10^{-6}	D3	101	10^{-7}	D2
83	10^{-6}	D2	78	10^{-5}	D3	103	10^{-7}	D2
81	10^{-5}	D4				107	10^{-6}	D2
88	10^{-5}	D2				111	10^{-6}	D2
						112	10^{-5}	D2
				Rats fed equivalent of 0.1 cc. culture				
48	10^{-8}	D3	45	10^{-8}	D3	75	10^{-8}	D3
63	10^{-8}	D2	46	10^{-8}	D3	74	10^{-8}	S
64	10^{-7}	D2	50	10^{-7}	D3	81	10^{-7}	D4
73	10^{-7}	D2	63	10^{-7}	D2	83	10^{-7}	D2
77	10^{-6}	D3	66	10^{-6}	D2	83	10^{-6}	D3
73	10^{-6}	D3	64	10^{-6}	D3	84	10^{-6}	S
90	10^{-5}	D2	69	10^{-5}	D2	91	10^{-5}	D2
84	10^{-5}	D2	72	10^{-5}	D3	93	10^{-5}	D2
			77	10^{-4}	D2	106	10^{-4}	D2
			72	10^{-4}	D3	103	10^{-4}	D4
			81	10^{-3}	D1	112	10^{-3}	D3
			92	10^{-3}	D2	120	10^{-3}	D2
			94	10^{-2}	D1			
			96	10^{-2}	D1			
				Rats fed equivalent of 1 cc. culture				
60	10^{-8}	S	46	10^{-7}	D3	73	10^{-8}	S
66	10^{-8}	D3	50	10^{-7}	D3	75	10^{-8}	S
80	10^{-7}	D3	64	10^{-6}	D6	80	10^{-7}	S
78	10^{-7}	S	65	10^{-6}	D3	81	10^{-7}	S
77	10^{-6}	D2	66	10^{-5}	D2	*88	10^{-6}	D8
85	10^{-6}	D3	67	10^{-5}	D4	89	10^{-6}	D2
86	10^{-5}	D2	75	10^{-4}	D3	95	10^{-5}	S
84	10^{-5}	D2	75	10^{-4}	D2	101	10^{-5}	S
			77	10^{-3}	D3	102	10^{-4}	D2
			81	10^{-3}	D2	104	10^{-4}	D2
			90	10^{-2}	D2	120	10^{-3}	D3
			80	10^{-2}	D3	121	10^{-3}	D2

TABLE XIII—*Concluded*

Test done 24 hrs. after feeding the filtrate			Test done 48 hrs. after feeding the filtrate			Test done 72 hrs. after feeding the filtrate		
Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
Rats fed equivalent of 5 cc. culture								
gm.	*cc.*		*gm.*	*cc.*		*gm.*	*cc.*	
59	10^{-8}	D3	45	10^{-7}	S	67	10^{-8}	S
67	10^{-8}	D3	58	10^{-7}	S	72	10^{-8}	S
70	10^{-7}	D4	68	10^{-6}	D4	82	10^{-7}	S
82	10^{-7}	D3	67	10^{-6}	D3	82	10^{-7}	S
85	10^{-6}	S	69	10^{-5}	S	89	10^{-6}	S
85	10^{-6}	D3	70	10^{-5}	D3	89	10^{-6}	S
86	10^{-5}	D3	75	10^{-4}	D2	92	10^{-5}	D2**
86	10^{-5}	D3	72	10^{-4}	D1	100	10^{-5}	S
			87	10^{-3}	D2	98	10^{-4}	D2
			86	10^{-3}	D1	104	10^{-4}	S
			87	10^{-2}	D2	110	10^{-3}	D4
			89	10^{-2}	D3	119	10^{-3}	D2
						126	10^{-2}	D1
Rats fed equivalent of 50 cc. culture								
58	10^{-8}	S	51	10^{-7}	S	*76	10^{-8}	D7
68	10^{-8}	D3	67	10^{-6}	S	83	10^{-8}	S
70	10^{-7}	D4	61	10^{-6}	S	87	10^{-7}	S
69	10^{-7}	D2	68	10^{-5}	S	94	10^{-7}	S
80	10^{-6}	D3	69	10^{-5}	D2	94	10^{-6}	S
67	10^{-6}	D5	70	10^{-4}	D2	101	10^{-6}	S
80	10^{-5}	D2	70	10^{-4}	S	101	10^{-5}	S
90	10^{-5}	D2	77	10^{-3}	D2	103	10^{-5}	S
			80	10^{-3}	D2	105	10^{-4}	D1
			83	10^{-2}	D1	110	10^{-4}	S
			86	10^{-2}	D1	110	10^{-3}	D7
						116	10^{-3}	D2
						119	10^{-2}	D1
Rats fed HCl acid killed pneumococcus equivalent to 50 cc. growth								
56	10^{-8}	D4	57	10^{-7}	D4	73	10^{-8}	S
68	10^{-8}	D2	63	10^{-6}	D3	74	10^{-8}	S
71	10^{-7}	D3	66	10^{-5}	S	82	10^{-7}	S
73	10^{-7}	D3	73	10^{-5}	D2	84	10^{-7}	S
77	10^{-6}	D3	73	10^{-4}	S	85	10^{-6}	S
73	10^{-6}	D3	77	10^{-4}	D2	89	10^{-6}	S
77	10^{-5}	D3	77	10^{-3}	D1	93	10^{-5}	S
88	10^{-5}	D3	78	10^{-3}	D3	106	10^{-5}	S
			81	10^{-2}	D2	110	10^{-4}	D2
			85	10^{-2}	D2	111	10^{-4}	S
						115	10^{-3}	D2
						118	10^{-3}	S
						137	10^{-2}	D2

* Pneumococcus in heart's blood.

** Mechanical injury during injection.

two survivors in the 1 cc. group in the 24 hour test, indicate that, had the 48 hour test not been irregular, a rather favorable degree of protection would have been

TABLE XIV

Comparative Immunizing Value of a Single Feeding Equivalent to 1 Cc. and 5 Cc. of HCl Acid Killed Pneumococcus Growth

48 hrs. after feeding			67 hrs. after feeding		
Wt.	Dose	Result	Wt.	Dose	Result
gm.	*cc.*		*gm.*	*cc.*	
*C86	10^{-9}	D3	*C62	10^{-9}	D2
C103	10^{-8}	D3	C81	10^{-8}	S
C105	10^{-8}	D5	C84	10^{-7}	D2
C111	10^{-7}	D2	C91	10^{-6}	D2
C106	10^{-7}	D2			
C118	10^{-6}	D2			
Rats fed equivalent of 1 cc.					
E66	10^{-8}	S	E51	10^{-8}	S
E52	10^{-8}	S	E69	10^{-7}	D2
E70	10^{-7}	S	E76	10^{-7}	S
E72	10^{-7}	S	E77	10^{-6}	D2
E72	10^{-6}	D3	E76	10^{-6}	S
E92	10^{-6}	D3	E88	10^{-5}	D2
E94	10^{-5}	D2	E85	10^{-5}	S
E95	10^{-5}	D2	E92	10^{-4}	D2
Rats fed equivalent of 5 cc.					
E38	10^{-7}	S	E51	10^{-7}	S
E45	10^{-7}	S	E54	10^{-7}	S
E54	10^{-6}	S	E57	10^{-6}	S
E54	10^{-6}	S	E57	10^{-6}	S
E55	10^{-5}	S	E57	10^{-5}	S
E60	10^{-5}	S	E57	10^{-5}	S
E75	10^{-4}	D4	E63	10^{-4}	D2
E70	10^{-4}	D2	E60	10^{-4}	S
E85	10^{-3}	S	E90	10^{-3}	D3

* Pneumococcus in heart's blood.

obtained with this quantity at that time. The fairly extensive protection which the rats in the 1 cc. group possessed in the 72 hour test shows that this opinion is justified, and that the correct dose lies between 1 and 5 cc. The 5 and 50 cc.

amounts gave the usual 72 hour results. The acid killed bacteria produced the most uniform protection, but the difference in survivals between this group and the 5 cc. filtrate and 50 cc. filtrate groups is insufficient to warrant the conclusion that the results are very different.

There still remained the question whether acid killed pneumococci equivalent to 5 cc. and 1 cc. of growth would yield the same protection as equal quantities of bile dissolved organisms.

To obtain data on this point an experiment was performed in which acid killed bacteria equivalent to 1 cc. and 5 cc. growth were fed. Feeding took place for the two groups simultaneously, from the same culture, and following a 20 hour fast. Half the animals of each set were tested 48 hours and the other half, 67 hours after the ingestion of the organisms. The results, given in Table XIV, show that the growth from 1 cc. and from 5 cc. produce a resistance against 100 and 10,000 or more fatal doses, respectively after 48 hours. After 67 hours, the rats fed 1 cc. seem to have a greater resistance than these from the same group examined the day before, but it is irregular. The animals in the 5 cc. group respond about the same as those tested after 48 hours.

The results, as a whole, are about the same as were obtained when the Berkefeld filtrate of bile salt dissolved pneumococci was fed in equivalent quantities, and seem to show that no serious loss of active material takes place when the solution passes through the filter. It would also seem that the bile salt plays no part in the process of immunization.

DISCUSSION

It is apparent from the data accumulated here, that considerably smaller quantities of pneumococci than were used in the early experiments are sufficient to produce a high degree of protection. The organisms from 5 cc. culture or possibly less, either intact or dissolved in sodium glycocholate are enough. The increased resistance to the virulent pneumococcus is present, probably to the full extent, or almost so, 48 hours after the material is ingested and can occasionally be detected to a smaller degree at the end of 24 hours. It would seem that once a sufficient amount is fed, no great advantage accrues from the use of a larger dose so far as degree of immunity is concerned, although there may be an effect on its duration. The promptness and ease with which the increased resistance is created, makes the method

attractive when considered as a possible practical means of protecting human beings, especially where it is essential that the immunity be built up quickly. For the same reason it may be that feeding the pneumococcus in the very early stages of pneumonia will prove of value in favorably influencing the course of the disease. No apparent discomfort has been found to follow the ingestion, by human beings, of quantities of acid killed pneumococci which are much larger than would be required if dosage is calculated on the basis of weight. Measurements of the protective value of the sera of such individuals before and after the ingestion of the bacteria showed no increase in a few, while several others showed increases equivalent to 1, 10 and 100 fatal doses. Earlier experiments (4) have shown approximately similar results with the sera of rats immunized by feeding acid killed pneumococci. Judging from the results obtained with rats, it is uncertain whether the immunity need in all individuals be accompanied by an increase in the protective value of the serum. When an increase does take place, it is a relatively small one. The results so far obtained with humans are encouraging and this phase of the work is being continued. Additional work is also being done to determine the effect of the size of the dose, number of doses, and intervals, on the duration of the immunity. Recent experiments have shown that a single feeding, although producing a high degree of protection, does not create a very durable one, but that when the effect has worn off another single feeding will result in the reappearance of the high resistance.

In judging the relative values of the various forms in which the bacteria have so far been administered, the following facts stand out. The use of degraded avirulent cultures of Type 1 is of little value, only an occasional animal having been protected. The use of milk cultures is effective and may prove of value as a form in which the bacteria can be administered to humans. Sedimented bacteria, either whole or disrupted, or solutions of the organisms, permit of smaller actual bulk being used. Judging by the very favorable results obtained with bile dissolved cocci, it appears possible that even when intact cells are fed, they may be disrupted in the gastro-intestinal tract, and that the active portion undergoes solution before absorption takes place.

The fact that the dissolved contents of the pneumococcus are effec-

tive in eliciting an immunity when fed, permits one to regard an ingredient of the cell, rather than the intact organism as the active material. The variety of treatments to which either the whole or the dissolved cell has been submitted and then tested, allows one to draw up a tentative list of some of the properties of this substance. The results with the degraded pneumococcus, Type 1, indicate that the antigen is formed in relatively small quantities in this kind of cell. The antigen is water soluble and is uninjured by bile salts. Since the milk cultures were found effective, it would seem that heating at 60°C. for an hour in the presence of the lactic acid formed, did not destroy it. Earlier experiments (2) showed that heating the pneumococcus at 80°C. for 2 hours caused considerable destruction, although some of the rats fed the heated bacteria tolerated many fatal doses indicating that not all of the active ingredient was lost by this treatment. Contact with "N/12" hydrochloric acid for 3 hours at about 30°C. does not destroy it. Neither does desiccation. It is not destroyed by the gastrointestinal enzymes of the white rat. Attempts are now being made to determine additional properties of the substance.

It is still too early to say whether the active ingredient is one of the definitely known antigenic components of the pneumococcus cell (protein, carbohydrate).* These two substances are supposed to exist united in an intact cell as a protein-carbohydrate complex. Solution of the cell is regarded as being accompanied by dissociation of the union and is known to destroy the property of eliciting agglutinins for the whole cell as well as precipitins for the carbohydrate fraction. The experiments described prove that solution does not interfere with the creation by feeding, of an increased resistance to the virulent organism. The few experiments which we have done showing that the protection is type specific (these need further support) might be interpreted as indicating either that a type specific substance other than the protein-carbohydrate complex is responsible or that this complex is not completely dissociated on solution and is responsible for the effects

* Experiments done since writing the above show that feeding the specific polysaccharide of pneumococcus, Type 1, produces an increased resistance to the organism. In degree and time of appearance this resistance resembles that obtained by the methods described in this article. So far, however, the proportion of animals protected is smaller.

observed. The inability to find agglutinins and precipitins in the sera of immunized animals would argue against the latter conception.

SUMMARY

1. Feeding heat killed pneumococci grown in milk produces a fair degree of immunity.

2. Feeding acid killed degraded avirulent organisms produces little protection.

3. Feeding the desiccated, mechanically disrupted organisms creates a high degree of protection.

4. Feeding the Berkefeld filtrate of sodium glycocholate dissolved cells produce a high degree of immunity.

5. A single ingestion of this material equivalent to between 1 and 5 cc. growth is sufficient to protect a rat against 1000 to 10,000 fatal doses. Among rats fed the equivalent of 0.1 cc. an occasional one survives.

6. This degree of protection is present 48 hours after the feeding, and to a smaller extent exists in occasional animals at the end of 24 hours.

7. The treated animals are resistant to subcutaneous as well as intraperitoneal injections.

8. A single ingestion of hydrochloric acid killed pneumococci equivalent to between 1 and 5 cc. growth also protects within 48 hours against 1000 to 10,000 fatal doses intraperitoneally injected.

9. Reference is made to results obtained in preliminary experiments with human beings.

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THE EFFECT ON THE RATE OF UREA EXCRETION OF CARMINE DEPOSITION IN THE CELLS OF THE RENAL TUBULES*

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A localized concentration of urea has been demonstrated in the epithelium of the proximal convoluted tubules of the kidney by Leschke (1) and this observation has since been confirmed by Oliver (2).

The method, which depends on the formation of an insoluble compound between urea and mercuric nitrate, is open to criticism on the basis of a possible lack of specificity. Chevallier and Chabanier (3) have found a similar high urea concentration in the cells of the convoluted tubules by means of a more delicate microchemical reaction, namely, the precipitation of dixanthyl urea by the interaction of urea and xanthydrol. Oliver (4) has repeated and extended the study of the distribution of urea in the kidney by means of the xanthydrol reaction. He found that extracellular crystals of dixanthyl urea were demonstrable in varying degree in all parts of the lumen of the renal vascular system. In the urinary lumen, sparse crystals were present in the glomerular capsule, more in the convoluted tubule, with still further accretions lower in the tubule. Crystals contained in cells were found only in the cortex, where the thick epithelium of the proximal convoluted tubule was filled with them. The number of crystals in the tubule cells gradually decreased so that there were very few in the terminal spiral portions of the proximal tubule and in the other divisions of the renal tubule intracellular crystals were nearly always absent. These observations have since been confirmed by a number of investigators (5, 6, 7) who have used the same method.

The question arises as to whether the source of this high urea concentration in the cells of the proximal convoluted tubules is absorption from the lumen of the tubule or excretion from the blood. In the light of other knowledge relating to the mechanism of urine formation

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Oliver (4) concluded that the second source was the more likely, since the assumption of an excretion by the tubule cells was the most direct conclusion from the evidence then at hand. More recently, however, the importance of absorptive activities has been emphasized by Oliver and Shevky (8) in experiments in simpler kidneys and with more direct methods, so that it would seem appropriate to reexamine the question. If it were possible to occlude the tubule cells or even decrease their function without affecting the other elements of the kidney the effects should indicate whether the tubules are removing urea from the urine by absorption or excreting it into the tubule lumen from the blood stream. The experiments which follow were therefore devised.

After the administration of a dye such as carmine by the vascular route, a fine granular deposit of the dye takes place in the tubule cells and, as has been shown by Suzuki (9), this deposit is most marked in the cells of the proximal convoluted tubules. It seemed possible that an excessive storage of the dye in these cells might interfere with their normal function. Just how much "blocking" there is may be questioned, but the histological appearance of these cells indicates that they have been damaged by the deposition of the dye and this alone might be expected to alter their normal activity. On the other hand, no histological evidence of damage to the other structures of the kidney can be found. Consequently the rate of urea excretion has been determined in a group of rabbits before and following intense staining of the renal tubule cells by intravenous injections of lithium carminate. Certain special conditions have been found (10) under which the rate of urea excretion in any one subject varies only with the blood urea concentration so that the excretory ratio: $\frac{\text{Rate of urea excretion}}{\text{Blood urea concentration}}$ becomes a constant. In the experiments to be described here any increase or decrease in the rate of urea excretion as a result of the experimental variable may then be measured by the changes in this "ratio."

Methods

Healthy male rabbits were used. After abstinence from food for 18 hours, 1 gm. of urea per kilo body weight was given in 4 per cent solution by stomach tube

and every hour thereafter for 5 hours the tube was again introduced and 15 ml. of water per kilo body weight were administered. Three hours after giving the urea the bladder was catheterized and washed. At approximately hourly intervals thereafter three collections of urine were made by catheterization and after each collection the bladder was washed out. Samples of blood were obtained by puncture of the left heart at the middle of these three periods of urine collection. The urea in the urine and the blood was determined by Addis' methods (11) and the average ratio: $\frac{\text{Urea in 1 hour's urine}}{\text{Urea in 100 ml. blood}}$ was calculated.

Beginning on the day that the first "ratio" was determined and every other day thereafter each rabbit was given 4 ml. per kilo of a 2.5 per cent* solution of carmine in half saturated lithium carbonate until a total of 8 doses containing a total of 0.8 gm. carmine per kilo had been given. Three days after the last carmine injection and 18 days after the first one, the rate of urea excretion was again determined by the same procedure as has already been described. The following is a typical protocol:

Rabbit 1. Weight, 2800 gm.
November 21, 1924.
12:30 p.m.—70 ml. of 4 per cent urea solution in water by stomach tube.
1:33 " —42 ml. of water by stomach tube.
2:31 " — " " " " " " "
3:33 " — " " " " " " "
4:36 " — " " " " " " "
5:32 " — " " " " " " "

The times given in the following table are those at which the washing of the bladder was completed and the times at which blood was collected.

Time	Urine urea	Time	Blood urea	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$
	mg. per kilo per hr.		*mg. per 100 ml.*	
3:30–4:32	257	4:00	120	1.30
4:32–5:30	148	5:02	105	1.41
5:30–6:30	148	6:00	94	1.57
Average (before carmine).....				1.42

* The carmine solutions showed peculiar differences. The first dye was a prewar product made in Germany. Excellent results were obtained with this but they could not be duplicated with domestic products of recent manufacture. The latter proved to be toxic and killed numerous animals but after the solution had remained in the laboratory in a loosely stoppered bottle for over 2 years it also was without obvious ill effects. When freshly prepared, the solution has a strong acrid odor resembling oil of cloves and this disappeared almost entirely on standing.

11.2 ml. of 2.5 per cent carmine in half saturated lithium carbonate solution were injected intravenously through the marginal ear vein on November 21, 23, 25, 27, 29, and December 1, 3, and 5.

December 8, 1924.

Urea solution and water were administered as on November 21.

Time	Urine urea	Time	Blood urea	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$
	mg. per kilo per hr.		*mg. per 100 ml.*	
3:35–4:36	189	4:05	130	1.38
4:36–5:34	192	5:06	116	1.64
5:34–6:35	196	6:06	112	1.76
Average (after carmine).....				1.59

RESULTS

The results of the 20 experiments are given in Table 1. In every animal except one the rate of urea excretion was greater after the renal tubules had been stained with carmine than before. In the single exception there was no appreciable change. There is considerable variation in the degree of increase in the rate of urea excretion for the different rabbits in the group but this might be expected as a result of the variable staining of the renal tubules, the kidneys of some rabbits containing appreciably more carmine than those of others. The actual average increase in the ratio: $\frac{\text{Rate of urea excretion}}{\text{Blood urea concentration}}$ was 0.17, a 13.8 per cent increase in the rate of urea excretion. Although not of large magnitude the increase considered from a statistical point of view is significant. The expected difference between the average ratios before the carminization and those obtained afterward is only 0.04, while the observed difference is 0.17. The chances then are that the increase is due to the known experimental variable. There may be some question concerning the applicability of such statistical method to data of the nature of those with which we are concerned here. However in two other groups of rabbits of 10 and 12 animals each, in which urea excretory ratios were determined as in the carmine experiments and repeated within about the same period of time but without the introduction of an experimental variable, the actual average change was plus 2 per cent for one group and minus 3 per cent for the other.

In practically every experiment the urine volumes during the three ratio periods were appreciably greater after the carmine injections than those obtained after the administration of the same amount of urea

TABLE 1

No.	Weight	Urine volume cc. per kilo per hour		Urea ratio: Urine urea rate / Blood urea conc.		Ratio increase	
		Before carmine	After carmine	Before carmine	After carmine	Actual	Per cent
	gm.						
1	2800	12.3	15.5	1.42	1.59	0.17	10.6
2	2700	16.4	23.9	1.36	1.50	0.14	9.3
3	2100	21.2	23.9	1.34	1.45	0.11	7.6
4	2200	20.9	35.2	1.35	1.80	0.45	33.3
5	2250	15.6	20.8	1.00	1.37	0.37	37.0
6	2500	14.8	14.5	1.18	1.38	0.20	16.9
7	2500	21.4	14.6	1.39	1.32	0.00	0.0
8	2400	16.6	20.0	1.24	1.39	0.15	12.1
9	2600	14.6	17.2	1.40	1.47	0.07	5.0
10	2600	14.6	15.2	1.00	1.10	0.10	10.0
11	3100	12.1	13.0	1.30	1.31	0.01	0.1
12	2200	11.4	14.7	1.39	1.46	0.07	5.0
13	2000	11.1	19.5	1.10	1.37	0.27	24.5
14	2000	12.2	13.7	1.02	1.38	0.36	35.2
15	1600	7.9	18.7	1.48	1.95	0.47	31.8
16	1600	6.0	12.9	0.89	1.03	0.14	15.7
17	1900	11.7	16.0	0.97	1.00	0.03	3.0
18	1900	12.3	17.0	1.25	1.49	0.24	19.1
19	2500	13.3	16.6	1.19	1.26	0.07	5.9
20	2500	9.7	15.3	1.23	1.30	0.07	5.7
Mean.....	2300	13.8	17.9	1.23	1.40	0.17	14.4
Standard deviation....		4.02	4.94	0.17	0.21		
Probable error.....		0.61	0.75	0.03	0.03		
Coefficient of variability.		29.1	27.6	13.9	14.9		
Actual difference of the means.....		4.10		0.17			
Probable difference of the means.....		0.96		0.04			

and water in the three 1-hour periods carried out before. Although it may have some significance this increased water excretion may be only secondary to the increased amount of urea excreted after the carmine injections.

DISCUSSION

The results do not support the idea that the high urea concentration in the cells of the proximal convoluted tubules represents urea passing from the blood into the tubules in the secretion of urea by these cells. On the contrary they lend weight to the theory that all of the urea comes from the glomerular filtrate and that part of it is absorbed by the tubules.

It has been demonstrated (12) in the perfused frog's kidney that urea is excreted by glomerular filtration and the evidence is strongly suggestive of tubular reabsorption. More indirect experiments showed that in all probability urea is likewise excreted through glomerular filtration by the mammalian kidney. This supports the view that the high urea concentration in the tubule cells represents urea reabsorption. Other experiments to be reported elsewhere, in which the excretory ratios of creatinine and urea were compared, also lend support to this contention.

Under certain "standard conditions," conditions which it is reasonable to suppose would be most apt to cause all of the renal elements to become active and result in a state of full renal activity, a remarkable constancy in the ratio: $\frac{\text{Urine urea rate}}{\text{Blood urea concentration}}$ has been demonstrated (13). This relation between the rate of urea excretion and the urea concentration in the blood is exactly what would be predicted if urea is eliminated entirely by glomerular filtration, provided that it is not reabsorbed and the rate of filtration is constant. In fact, if no reabsorption of urea takes place under the "standard conditions," the ratio $\times 100$ would represent the amount of glomerular filtrate in cubic centimeters per hour. Although we are presenting evidence which points to a reabsorption of urea, certain observations (14) have been interpreted to indicate that urea might be reabsorbed ordinarily but that under the "standard conditions" this urea reabsorption disappears. It is, however, under the "standard conditions" that urea reabsorption appears to have been demonstrated. It then becomes necessary to reconcile these observations. To do this one must assume that if urea is reabsorbed, the reabsorption rate under "standard conditions" is directly proportional to the blood urea concentration,

or consequently the observed urine urea rate, an hypothesis which after all is not unreasonable.

SUMMARY

The evidence that urea is present in high concentration in the cells of the convoluted tubules of the kidney and is passing through these cells has been reviewed. Following the deposition of a large amount of carmine in the cells of the convoluted tubules the rate of urea excretion, as measured by the relation of the rate of urea excretion to the blood urea concentration, is increased. This is interpreted as due to blockage or damage to the cells of the convoluted tubules which interferes with their usual function of absorption of urea and other substances from the glomerular filtrate and hence increases the amount of urea which reaches the bladder urine at a given blood urea concentration.

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STUDIES ON THE COMMON COLD

III. The Upper Respiratory Flora of Infants

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In recent years investigators of the common cold have realized the importance of ascertaining the normal basal flora of the human nasopharynx before drawing any conclusions as to the rôle played by any given microörganism in disease. From the observations of Williams (1), Bloomfield (2) and Jordan (3), it became apparent that many of the bacteria formerly thought to be responsible for the production of colds might occur as seemingly harmless inhabitants of the throats of healthy adults. Shibley, Hanger and Dochez (4), studying a group of thirteen normal adults throughout an entire winter and comparing their basal flora with that occurring during their colds, arrived at the conclusion that the potential pathogens may be present in healthy throats, that no bacteria can be found in early cold cultures to which an etiological rôle can be assigned, but that certain organisms probably play a part as late or secondary invaders. Exception may possibly be noted, however, in the case of certain geographically unusual epidemics as described by Burky and Smillie (5); in the metropolitan centers, on the other hand, these conclusions, supported later by Noble, Fisher and Brainard (6), hold good. By inference, then, the cause of the acute infectious cold, remaining undetected by ordinary bacteriological methods, probably falls into the category of an unknown virus to be sought by the methods of Foster (7), Olitsky and Gates (8), and later by Dochez, Shibley, and Mills (9).

The present study of the nose and throat flora of infants was undertaken with two ends in view: in the first place, no observations on the bacteriology of the pharynx of the newborn could be discovered in the literature, and secondly it was felt that the importance of the various bacteria could be more clearly evaluated if studied in the simplest possible host. The infant, unaltered by long contact with a hostile environment, seemed an ideally simple medium for the study of upper respiratory disease.

Methods.—Cultures of the nose were made by passing a sterile cotton swab on fine copper wire through the naris as far as possible; those of the throat were obtained by swabbing the visible portion of the oropharynx. Swabs were immediately rubbed on freshly-poured 5 per cent rabbit's-blood-agar plates and streaked with a platinum wire. The plates were incubated aerobically and read at the end of 20 to 24 hours. Identification of the organisms was made by colony appearance, staining and morphology, with more complete bacteriological study where indicated. Diphtheroid bacilli were taken as a group, as were the Gram-negative cocci. Streptococci were classified as green, hemolytic and indifferent (alpha, beta, and gamma types). Members of the colon group were tested for sugar fermentation. All pneumococci were typed and injected into a mouse to obtain a rough measure of virulence, those strains of which 0.1 cc. of an 18 hour blood broth culture killed being considered virulent. Staphylococci were listed according to pigment formation and a large staphylococcus-like organism growing in gray colonies was designated "large Gram-positive coccus." Pfeiffer's bacillus was carefully searched for and, if hemolytic, distinguished as "Bacillus X."

TABLE I

First Positive Throat Culture—Predominating Organism

30 newborn babies

Green streptococcus	Indifferent streptococcus	*Staphylococcus albus*	*Staphylococcus aureus*	Colon group	Diptheroid group	Large Gram-positive coccus
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
37	23	2.7	3	3	3	3

Results.—Observations were first made on 30 newly-delivered babies in Sloane Hospital for Women. In the beginning of the series, cultures of the oral contents were actually obtained in the delivery room, but when these were found to be almost invariably sterile the practice was discontinued and the first throat culture was made a few hours after birth. In 80 per cent of cases these initial throat cultures showed no growth and until the first feeding the pharynx ordinarily remained sterile. After the first feeding, however, the initial flora had regularly made its appearance and the pharynx never regained its original sterility. The first predominating organism to appear was ordinarily the non-hemolytic streptococcus (60 per cent), with staphylococci next in frequency (30 per cent). Table I shows the relative frequency with which these organisms predominated in the first positive cultures to be obtained.

Successive cultures were then made during the first 2 weeks of life, that is to say, during the infant's stay in the maternity hospital. Profuse growth was almost always obtained from the throat. Over the whole series of these cultures indifferent streptococci were more prominent than the green producing type. *Staphylococcus albus* was almost always present, less frequently predominant than indifferent streptococci, but more frequently than green. In order of diminishing importance the following organisms were also observed: large Gram-positive cocci, *Staphylococcus aureus*, colon bacilli, diphtheroid bacilli, and Gram-negative cocci (see Tables III and IV). Pfeiffer's bacillus, pneumococcus and hemolytic streptococcus were never observed in the entire group in the first 2 weeks of life—in spite of the fact that during the whole course of the study the incidence of Pfeiffer's bacillus in the

TABLE II

First Positive Nasal Culture—Predominating Organism

25 cases

Staphylococcus albus	*Staphylococcus aureus*	Diptheroid group	Large Gram-positive coccus	Indifferent streptococcus	Green streptococcus
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
76	8	4	4	4	4

normal hospital population ran well over 50 per cent. Thus the basal flora of the normal infant's pharynx in its first 2 weeks of life presents one striking difference from that of the normal adult's: the potential pathogens are never found. An additional note is the relatively much lower incidence of the Gram-negative cocci.

Nasal cultures were also made and showed no growth on the first day of life in 85 per cent of cases. In 40 per cent they remained sterile on the second day as well. Following this, organisms were invariably present, although growth from the nose, when established, was rarely abundant. The organism to predominate in the first positive culture was the *Staphylococcus albus* in most instances.

During the first 2 weeks of life the staphylococci were by far the most prominent organisms in the nose. Diphtheroid bacilli ranked next in incidence with large Gram-positive cocci following. Strepto-

cocci were much less common than in the throat. Colon bacilli were quite infrequent (see Tables V and VI). As in the throat cultures, the potential pathogens were never found and the same differences existed between the nasal cultures of the newborn and the normal adult as were observed in the throat. That is to say, pneumococcus, Pfeiffer's bacillus and hemolytic streptococcus were never present in the nose during the first 2 weeks of life.

TABLE III

Percentage Incidence of Different Organisms in Throat Cultures

	Indiff. strep.	*Staph. albus*	Green strep.	Large Gram-pos. cocci	*Staph. aureus*	Colon group	Diphtheroid bac.	Gram-neg. cocci	*B. pfeifferi*	Pneumococcus	Hem. strep.	Bacillus "X"
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
A. 30 normal infants during first 2 weeks of life No. of cultures, 155	78	92	44	25	23	16	7	4	0	0	0	0
B. 28 normal infants between 4 and 8 months of age	61	46	100	36	18	7	7	71	53	0	11	7
C. 21 infants early in the course of their first cold Average age, 3 months	57	62	91	38	24	10	24	38	14	0	0	0
D. 14 infants with recurrent colds Average age, 7 months	21	14	100	29	7	0	21	71	57	14	0	7

Following discharge from the maternity wards, as many of these infants as possible were followed from time to time, particularly with a view to obtaining cultures during their first colds. In addition, other dispensary babies were cultured and the combined findings of the entire series are given in Tables III, IV, V, and VI, showing percentage incidence and percentage predominance of the various organisms.

The infants were divided into four groups. Group A—normal infants in the first 2 weeks of life—has already been discussed.

Group B is next introduced for comparison to show what changes take place as the infant grows older. It consists of 28 babies from 4 to 8 months of age, all entirely free from colds. Certain important differences are at once apparent in the throat cultures. For the first

TABLE IV

Percentage Predominance of Different Organisms in Throat Culture

	Indiff. strep.	*Staph. albus*	Green strep.	Large Gram-pos. cocci	*Staph. aureus*	Colon group	Diphtheroid bac.	Gram-neg. cocci	*B. pfeifferi*	Pneumococcus	Hem. strep.	Bacillus "X"
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
A. 30 normal infants during the first 2 weeks of life No. of cultures, 155	41	32	19	5	1	5	1	0	0	0	0	0
B. 28 normal infants between 4 and 8 months of age	7	0	78	0	0	0	0	14	3	0	0	0
C. 21 infants early in the course of their first cold Average age, 3 months	14	0	76	0	0	0	0	0	10	0	0	0
D. 14 infants with recurrent colds Average age, 7 months	14	0	86	0	0	0	0	0	0	0	0	0

time the potential pathogens were found. It will be observed that Pfeiffer's bacillus was present in the majority of these normal infants' throats (53 per cent), and that hemolytic streptococci were noted in 11 per cent. The throat cultures also showed an increase in prominence of the green-producing streptococcus and the Gram-negative cocci, at the expense of indifferent streptococci.

In the nose two potential pathogens also made their first appearance

and while *B. pfeifferi* was rare, pneumococci appeared in 14 per cent of the cultures and in 11 per cent were actually predominant. (Nine of the infants never had colds; the remaining 19 gave a history of one or more. No significant differences in the flora were noted except a slightly higher incidence of Gram-cocci in the throats of the latter.) The significance of the changes observed in this group is interesting; it appears that in the first 6 months of life the newborn's flora steadily approaches that of the normal adult and that infants can actually harbor

TABLE V

Percentage Incidence of Different Organisms in Nasal Cultures

	Staph. albus	Diphtheroid bac.	*Staph. aureus*	Large Gram-pos. cocci	Indiff. strep.	Green strep.	Colon group	Gram-neg. cocci	*B. pfeifferi*	Pneumococcus	Hem. strep.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
A. 30 normal infants during first 2 weeks of life No. of cultures, 103	93	43	32	21	15	4	3	1	0	0	0
B. 28 normal infants between 4 and 8 months of age	79	46	22	68	14	7	7	18	7	14	0
C. 21 infants early in the course of their first cold Average age, 3 months	62	81	48	43	14	10	5	14	14	0	0
D. 14 infants with recurrent colds Average age, 7 months	57	29	21	57	7	7	14	14	21	43	0

the pathogens with impunity. In other words, the mere acquisition of a potentially pathogenic organism by an infant does not necessarily produce any symptoms whatsoever. Pneumococcus, Pfeiffer's bacillus, and hemolytic streptococcus can apparently live as harmless inhabitants of the upper respiratory tract of an entirely normal infant in the first 6 months of life.

Group C represents 21 infants studied early in the course of their first cold. From a clinical standpoint these first colds are usually very mild.

The child sneezes and coughs; there may or may not be a small amount of thin nasal discharge and constitutional symptoms, as a rule, are absent. There is no real difference between the flora found in these first mild colds and that of the normal group. The potential pathogens, while present, were actually less conspicuous than in the normals, a fact possibly attributable to the younger average age of these infants. In other respects the findings are strikingly similar; to be sure, the incidence of diphtheroid bacilli in the nose was high in the cold group, but it predominated less frequently. From this comparison it

TABLE VI

Percentage Predominance of Different Organisms in Nasal Cultures

	Staph. albus	Diphtheroid bac.	*Staph. aureus*	Large Gram-pos. cocci	Indiff. strep.	Green strep.	Colon group	Gram-neg. cocci	*B. pfeifferi*	Pneumococcus	Hem. strep.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
A. 30 normal infants during first 2 weeks of life No. of cultures, 103	67	5	18	5	4	0	2	0	0	0	0
B. 28 normal infants between 4 and 8 months of age	25	22	7	22	4	0	4	7	0	11	0
C. 21 infants early in the course of their first cold	19	10	24	24	10	0	5	0	5	0	0
D. 14 infants with recurrent colds	21	7	7	7	0	0	0	7	14	36	0

becomes apparent that one cannot usually assign a causative rôle to any organism in the first colds of infancy, and it might be justifiable to conclude that the process is similar to the acute infectious cold in the adult, mention of which has already been made.

Group D, composed of 14 infants, on the average slightly older (7 months), includes only those who gave a history of recurrent colds, cultures being taken during an exacerbation or reinfection. Clinically speaking, many of these infants were quite separate from the "first cold" group; their symptoms were often severe, with purulent nasal discharge, loss of appetite and fever. Their throat flora, however,

was similar to that of the normal group excepting for the first appearance of pneumococci (14 per cent). On the other hand, a striking change was noted in the nose. Pneumococci were found in 43 per cent, actually predominating in 36 per cent. Pfeiffer's bacillus was noted in 21 per cent and it predominated in 14 per cent. Most of the infants showing a heavy growth of either of these organisms from the nose presented a picture similar to that of chronic winter colds in adults, with purulent nasal discharge and the severer symptoms mentioned above. It is not improbable that this represented a secondary invasion of the respiratory tract by these pathogenic organisms, with chronic lodgment, perhaps in the maxillary antrum, and frequent exacerbations precipitated by various causes. (The pneumococci fell into Group IV and most of them were "virulent.")

CONCLUSIONS

1. The upper respiratory tract is sterile at birth.

2. In the first 2 weeks of life the infant acquires a basal flora comparable to that of adults except that the potential pathogens are absent.

3. During the ensuing months the potential pathogens may appear without giving rise to symptoms and by 8 months the infant's flora is entirely comparable to the adult's.

4. There is no evidence of a specific bacterial incitant for the first colds of infancy.

5. In infants with recurrent colds, secondary infection of the nose with pneumococci or *B. pfeifferi* probably plays a part.

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REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

IV. The Development of Skin Reactivity to Derivatives of Pneumococcus

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Many reports have been made concerning the occurrence of reactions in the skin following the intracutaneous injection of Pneumococcus products in pneumonia patients at the time of crisis, or after the crisis, or into guinea pigs after they had previously received injections of the organism or its derivatives. These reports indicate that the reactivity of the skin to certain derivatives of Pneumococcus may become increased after the patient or animal has undergone infection, or after they have been injected with certain products of Pneumococcus parenterally. It has usually been assumed, therefore, that a state of increased reactivity of the skin to Pneumococcus protein may occur, probably analogous to the state of hypersensitiveness which arises following the administration of such a foreign protein as egg albumin. The exact conditions under which this state of altered reactivity of the skin may occur are not as yet well understood. Moreover, the relation of this condition of increased skin reactivity to the general state of altered reactivity or "allergy" is still as obscure in the case of pneumococci and their products as it is in the case of most other reacting agents. An understanding of the underlying principles can probably be arrived at only after the various phenomena have been carefully observed and recorded under a great variety of conditions and in various animals. Experience has shown that the various phenomena thought to be related to the general condition of hypersensitiveness, such as reactions occurring in the skin following the injection of foreign substances, the ophthalmic reaction, anaphylaxis in the guinea pig, increased reaction

of the musculature of the uterus, serum sickness, etc., usually cannot all be elicited in the same animal. It is also probable that they are not necessarily all manifestations of the same biological process.

In the present study an investigation has been made of the skin reactions in rabbits following the injection of certain derivatives of the pneumococcal cells.

This work is a part of the investigation of the changes which take place in rabbits following the repeated intracutaneous injections of suspensions of heat-killed pneumococci, some of the results of which have been reported in previous communications (1, 2, 3). Consequently most of the skin tests have been made in rabbits that have been previously treated in this way, but for the sake of comparison, skin tests have also been performed in normal rabbits and in rabbits treated in various other ways. The derivatives of pneumococci employed in the tests have been the so-called "nucleoprotein," the supernatant fluid after precipitation of the "nucleoprotein" from the bacterial extract and to a less extent the carbohydrate fraction, the specific polysaccharide. The method of preparing the first two derivatives was given in a previous communication (1). The specific polysaccharides were obtained from Dr. Avery and the method of their preparation has been fully described (3). The rabbits were prepared by depilitation of the skin over the abdomen as previously reported (1).

The first skin tests were made in rabbits which had received repeated intracutaneous injections of suspensions of heat-killed pneumococci. About 3 weeks after the animal had received the last of 8 to 12 intracutaneous injections, tests were performed by injecting into the skin a solution of "nucleoprotein." The volume of fluid injected for each test was 0.2 cc. and the amount of the test substance was measured by the determination of the nitrogen present, considering this as all derived from protein. Control tests have been made repeatedly in normal animals that had received no preliminary treatment, and in none of these animals have reactions in the skin been observed following the injection of amounts of "nucleoprotein" two to four times larger than any doses employed in testing the previously treated animals.

It has been found that when 0.2 cc. of a solution of "nucleoprotein" is injected into the skin of a rabbit which has previously received intracutaneous injections of heat-killed pneumococci, within a few hours the skin about the site of injection becomes red, raised and edematous. The reactions reach a maximum in 24 to 48 hours, then begin to fade

and usually disappear within 3 to 4 days. The degree of reaction depends to a considerable extent on the amount of the protein contained in the solution. Reactions may occur with amounts of protein as small as 0.0025 mg. Usually larger amounts 0.2 to 0.6 mg. have been injected. Under the latter circumstances the area of skin involved in the reaction measures on an average 1.5 to 2 cm. in diameter. A certain area of erythema not infrequently is seen but a breaking down of the skin has never been observed.

The reactivity of the skin to the supernatant fluid after precipitation of the "nucleoprotein" from the bacterial extract was also studied in rabbits following repeated intracutaneous injections of heat-killed pneumococci. A solution of the concentrated bacteria was made by repeated freezing and thawing. The "nucleoprotein" was then precipitated out with acetic acid and the resulting supernatant fluid, adjusted to slightly acid to litmus, was boiled over a free flame for 10 minutes. This extract gave none of the usual qualitative tests for protein. In the doses employed in this study, it was found to possess a primary toxicity, so that in over half the normal rabbits, it caused a local reaction in the skin consisting of an erythematous blush with thickening. In these animals the reaction disappeared in 1 to 3 days. In rabbits which had previously received repeated intracutaneous injections, the skin reaction following the administration of this extract was more marked. The area of the skin involved was larger, the elevation was more marked, and a central area of ecchymosis, which was never seen in the untreated animals, was not infrequently observed. In some instances the skin over the lesion broke down. Moreover, in these animals the reactions lasted longer, disappearing in 3 to 5 days.

In some of the rabbits the reactivity of the skin to the specific soluble substances, the polysaccharides, was also studied. Dilutions of 1–1000, 1–10,000 and 1–25,000 of the carbohydrates were injected in the skin about 3 weeks after the last intracutaneous injection of heat-killed pneumococci. No reactions were seen following the intracutaneous introduction of the carbohydrate derived from homologous or heterologous types of Pneumococcus.

In terms of bacterial specificity, then, the skin reactions observed in rabbits after repeated intracutaneous injections of heat-killed

pneumococci are not type-specific, but species-specific. The skin reactions to the derivatives of Pneumococcus are elicited by materials which are not associated with type-specificity, and in all instances, the test reagents were derived from a type other than that employed during the repeated injections.

The Occurrence of the Skin Reaction Following Repeated Injections, by Different Routes, of Pneumococci or Their Products

Experiments were next undertaken to determine whether rabbits which have received repeated intravenous or intraperitoneal injections of dead pneumococci exhibit an increased reactivity of the skin, just as do animals which have received repeated intracutaneous injections.

TABLE I

The Incidence of the Skin Reaction to Pneumococcus Protein in Rabbits Following Repeated Injections of Pneumococcus or Its Derivatives

Material injected	Route of injection	Number of rabbits	Skin test	
			Positive	Negative
Heat-killed Pneumococcus	Intracutaneous	60	60	—
Heat-killed Pneumococcus	Intravenous	40	40	—
Heat-killed Pneumococcus	Intraperitoneal	2	2	—
Nucleoprotein	Intracutaneous	8	8	—
Nucleoprotein	Intravenous	8	8	—
Supernatant fluid from bacterial extract	Intracutaneous	4	4	—

It was found that in the former animals, skin reactions of the same nature might follow the injections of the derivatives of pneumococci. The data are presented in Table I. It is seen that 40 rabbits received intravenous administration, and two, intraperitoneal injections of heat-killed bacteria. All the rabbits were found to be skin reactive.

While skin reactions to the nucleoprotein were obtained with regularity in this group of rabbits following intravenous injections of pneumococci, it has been found that when skin tests are undertaken, as here, 3 weeks after the last injection of organisms, the skin reaction may not be of great severity or in some cases it may be absent.

In another experiment, the skin reactivity was studied in rabbits which had previously received repeated injections of the "nucleoprotein," also in rabbit which had received repeated injections of the supernatant fluid after precipitation of the "nucleoprotein" from a bacterial extract. The data of this experiment are given in Table I. It is seen that 8 animals received the "nucleoprotein" intracutaneously and 8, intravenously, while 4 rabbits received the supernatant fluid from the bacterial extract intracutaneously. The animals were tested for skin reactivity 19 days after the last injection of these materials. Skin reactions, as described above were obtained in all the animals.

It may be concluded, therefore, that regardless of the material employed for the injections and irrespective of the route of administration of the material, skin reactivity resulted in all the rabbits.

Relation of the Skin Reaction to Resistance to Infection

In a previous publication (3), it was shown that following repeated intracutaneous injections of heat-killed pneumococci, rabbits acquire a marked degree of resistance to infection by Pneumococcus of homologous or heterologous types. It was also shown that following similar injections with the "nucleoprotein" and supernatant fraction of the bacterial extract of Pneumococcus, rabbits do not become resistant to infection. In the present study it has aready been pointed out that skin reactivity to derivatives of Pneumococcus is found to occur in rabbits which have been injected by any route with the intact cell or its protein derivatives. It is obvious, therefore, that skin sensitivity occurs with equal frequency in animals which are resistant to infection (*i.e.*, following injections with the intact cell) and in nonresistant animals, as for example, following repeated injections with derivatives of Pneumococcus.

Relation of the Skin Reaction to Circulating Antibodies

It has already been stated above that the skin reaction described in this communication cannot be related to type-specificity. The skin reactions are elicited by a species-specific reagent in the absence of demonstrable type-specific antibodies, as is seen, for example, in the rabbits which receive repeated intracutaneous injections of heat-killed pneumococci (1). On the other hand, the skin reaction to the "nucleo-

protein" always occurs in the presence of circulating species-specific antibodies, and in a general way the intensity of the reaction to a given quantity of protein varies with the titre of species-specific antibodies in the sera of the animals.

DISCUSSION

That increased reactivity of the skin to Pneumococcus or its products may be induced artificially in animals or may accompany natural infection by Pneumococcus has been pointed out by a number of authors.

Mackenzie and Woo (1) described the development of a skin reaction to bacterial protein following intracutaneous injections of an alkaline extract of Pneumococcus. Zinsser and Tamiya (12) reported that guinea pigs sensitized by injections of pneumococci or the bacteria protein gave skin reactions to autolysates and they concluded that this reaction was not related to type-specificity. Later Zinsser and Grinnell (13) induced skin sensitization in guinea pigs to autolysates of Pneumococcus by previous inoculations of the same material. More recently Bull and McKee (14) demonstrated that after recovery from infection induced by intranasal instillation of live pneumococci, rabbits become highly skin sensitive to an autolysate of pneumococci. These authors also reported that such rabbits die of shock following intravenous injection of the same material.

Skin reactions in patients suffering with pneumonia have also been reported. Clough (6) showed that pneumonia patients reacted locally when a protein extract of the organism was injected into the skin, but this reaction did not vary materially from the one occurring in normal individuals. Weil (15) described a skin reaction in patients to an autolysate of Pneumococcus which begins to appear at about the time of crisis. Steinfeld and Kolmer (16) reported the occurrence of a skin reaction following intracutaneous injections of heat-killed bacteria in 30 per cent of pneumonia patients after crisis. Later Weiss and Kolmer (17) showed that patients with pneumonia gave skin reactions to pneumotoxin (a sodium choleate solution of the cell) from the fifth to the thirteenth day of the disease. Bigelow (18) also obtained skin reactions in pneumonia patients with heat-killed pneumococci and several soluble derivatives. Herrold and Traut (19) found that 73 per cent of pneumonia patients failed to react to injections into the skin of a filtrate of a 5-day culture of Pneumococcus, while only 15 per cent of normal persons failed to react. Poole, Bumstead and Blake (21) obtained skin reactions with a protein extract of Pneumococcus in patients after crisis. Tillett and Francis (20) have recently demonstrated that patients with pneumonia react locally to injections of the protein-free carbohydrate of Pneumococcus. The reaction occurs at about the time of crisis, is type-specific, immediate, and is of the wheal and erythema variety. They also obtained delayed skin reactions in patients with the

nucleoprotein of Pneumococcus. Except for immediate reactions to the carbohydrate described by Tillett and Francis the skin reactions described by all these observers followed injections of whole bacteria or protein derivatives and the reactions were of the "delayed" type.

In the present paper is reported the occurrence of skin reactions in rabbits to derivatives of Pneumococcus. It has been found that following repeated intracutaneous injections either of heat-killed Pneumococcus, or the "nucleoprotein," or of the supernatant liquid after the "nucleoprotein" has been precipitated from a bacterial extract, rabbits become skin reactive. The skin reactions are of the delayed type and may be elicited by injections of "nucleoprotein," or of a solution of the cells from which both acid precipitable and heat coagulable proteins have been removed. This skin reactivity, however, is not elicited by the specific carbohydrate of Pneumococcus.

The skin reactivity was found to occur in rabbits following repeated injections by the intravenous or intracutaneous route of the bacteria, or the protein derivatives of Pneumococcus. The skin reactivity to Pneumococcus protein appears to be unrelated to type-specificity, or to resistance of the animal to infection. On the other hand, the occurrence of the skin reactivity appears to be associated with the presence of circulating species-specific antibodies.

SUMMARY AND CONCLUSIONS

1. A skin reaction elicited by the injection of the Pneumococcus "nucleoprotein," or of a solution of the cells from which the acid and heat-coagulable proteins have been removed is described in rabbits which have previously received repeated intracutaneous injections of heat-killed pneumococci.

2. In terms of bacterial specificity, the skin reaction is considered to be not type-specific, but species-specific.

3. A similar skin reaction to the proteins of Pneumococcus occurs in rabbits following the repeated administration by the intravenous or intracutaneous route of the heat-killed organisms or their protein derivatives.

4. The skin reaction may occur independently of resistance to infection.

5. The skin reaction appears to be related to the presence of circulating species-specific antibodies.

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REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

V. The Development of Eye Reactivity to Derivatives of Pneumococci

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The present paper describes the development of eye reactivity to certain products of Pneumococcus as a result of repeated intracutaneous injections of the heat-killed bacteria. For the purpose of comparison, additional rabbits were given injections of the cells or their derivatives by other routes and the reactivity of the eyes in these animals was also studied.

EXPERIMENTAL

Methods.—The injection of rabbits with Pneumococcus or its derivatives was carried out as described in a previous communication (1).

Eye Test.—Eye tests were done according to the technique described by Derick and Swift (3, 4). The cornea was anesthetized with one drop of 10 per cent novocaine, and then it was lightly scarified with a cataract knife. Three scratches were made on one of the upper quadrants of the cornea beginning 1 to 2 mm. from the sclerocorneal junction and reaching to the center. One drop of the reagent was instilled into the conjunctival sac and the material was rubbed gently over the surface of the cornea by manipulation of the lid.

Rabbits received from 8 to 12 intracutaneous injections of bacterial suspension. About 20 days after the last injection eye tests were performed by dropping into the eye, prepared as previously described, one drop of a solution of "nucleoprotein" (2). For purpose of control, two drops of the same solution were dropped into one of the eyes of each of a series of normal rabbits.

In the normal controls no changes in the eyes were observed. In certain of the animals which had previously received intracutaneous injections definite reactions occurred. These reactions were of two kinds. In the less severe type of reaction there appeared, after

several hours, a more or less extensive injection of the vessels of the palpebral and scleral conjunctivae without any change in the cornea. The maximum reactivity was attained within 24 to 48 hours and the reaction usually lasted 3 to 5 days.

In the severe type of reaction, in addition to the conjunctival injection, there developed on the first or second day a turbidity over the scarified area on the cornea. On the third or fourth day, in certain cases, small loops of blood vessels, a pannus, began to appear near the sclerocorneal margin and the vessels grew downward toward the center. Occasionally, there appeared to be hemorrhage into the cornea surrounding the newly formed blood vessels. There was frequently a certain degree of edema of the conjunctivae, and in the more severe reactions, there was present in the conjunctival sac a mucopurulent

TABLE I

The Incidence of the Ophthalmic Reaction in Rabbits Following Repeated Intracutaneous Injections of Heat-Killed Pneumococci

Total number tested	Number giving eye reaction			Number giving no eye reaction
	Conjunctival	Corneal		
		With pannus	Without pannus	
60	8	15	32	20

discharge which was proved to be bacteria-free by smears and cultures. The general corneal turbidity did not last so long as the pannus, and the eye appeared normal again within 7 to 12 days, the duration depending upon the severity of the reaction. It will be noted that the corneal reaction described here resembles very closely the ophthalmic reaction reported by Derick and Swift (4) in their studies on hypersensitiveness of rabbits to non-hemolytic streptococci.

In Table I, a summary has been made of the occurrence of the eye reaction to nucleoprotein in rabbits which had received repeated ininjections of heat-killed pneumococci. It is seen that of 60 rabbits tested, in 40 an eye reaction occurred, in 20 there were no reactions. Of the 40 animals showing an eye reaction, in 8 the reaction was limited to the conjunctivae, in 32 the cornea was involved, and in 15 of these

a pannus developed. Experiments were then conducted to determine whether the eye reactions were elicited by a solution of pneumococci from which the acid and the heat coagulable proteins had been removed. As in the preceding experiments, a series of rabbits were given repeated intracutaneous injections of solutions of heat-killed pneumococci. These animals were then tested by placing in the eye one drop of the Pneumococcus solution. Two drops of the solution were also placed in the eye of each of a series of control rabbits. It was found that while the eyes of normal rabbits were unaffected by this material, eye reactions occurred in treated animals, but not with so great a regularity as with "nucleoprotein."

In other rabbits the reactivity of the eye was tested by employing the soluble specific substances of Pneumococcus. The carbohydrates were used in dilutions of 1–1000, 1–10,000, and 1–25,000, but eye reactions were not observed in any of the animals tested with the homologous or heterologous carbohydrates.

Since Derick and Swift elicited the ophthalmic reaction described by them by the instillation into the eyes of living bacteria, experiments were conducted to determine whether the increased eye reactivity following the repeated intracutaneous injection of pneumococci could also be demonstrated by the instillation of living organisms. As the instillation of living S pneumococci incites an infection which may obscure the reaction, living R cells were employed. An R strain was selected which was derived from a type of Pneumococcus other than that used for the preparatory injections. After scarification of the cornea of each of a series of rabbits that had previously received repeated intracutaneous injections of a suspension of heat-killed pneumococci, one drop of the living culture, concentrated 25 to 30 times, was transferred into the conjunctival sac. In no instance, however, was an eye reaction observed following the instillation of living R pneumococci.

The Rate of Development and Duration of the Eye Reactivity

In the following experiment the rate of development of eye hypersensitiveness in rabbits previously injected intracutaneously with suspensions of heat-killed Pneumococcus was studied. Fourteen rabbits were given one or more injections each. The rabbits were divided

into seven groups of two each, and the animals of every pair received the same number of inoculations. The animals of the various pairs received a different number of inoculations, those of the first pair receiving 1 injection, those of the last pair 7 injections. Three weeks after the final injections, the rabbits were tested for eye reactivity to "nucleoprotein." The results of this experiment are given in Table II. It is apparent that there is no regularity between the development of the eye reactivity and the number of intracutaneous injections of bacterial suspension. Thus, both rabbits receiving only 1 inoculation of bacterial emulsion showed severe eye reactions, while the two

TABLE II

The Development of Eye Reactivity in Rabbits Injected Intracutaneously with Heat-Killed Pneumococcus

Number of rabbits	Number of injections	Results of eye reaction			
		Negative	Positive		
			Conjunctival	Corneal	
				With pannus	Without pannus
2	1	0	0	2	0
2	2	0	1	1	0
2	3	1	0	1	0
2	4	1	0	0	1
2	5	0	0	1	1
2	6	2	0	0	0
2	7	0	0	1	1

that received 6 injections showed no reactions; and again, eye reactions were obtained in both rabbits which had received 7 inoculations of pneumococci in the skin. This irregularity indicates that individual rabbits vary greatly in the manner in which they react to intracutaneous injections of pneumococci, especially as regards the development of eye hypersensitiveness.

In another experiment, the duration of the eye reactivity was studied. It was found that there is considerable variation in different animals. The reactivity of the eye may last as long as 4 months; in two rabbits, the eye reaction was elicited 6 months after the last intracutaneous injection of bacterial suspension.

During the course of this study, rabbits which had previously been shown to be eye sensitive but in which the eye sensitiveness had later disappeared were subsequently injected with "nucleoprotein" solution intravenously or by some other route. It was frequently noted under these conditions that although the cornea was not scarified and nothing was instilled into the eye, following the subsequent injection of the protein, the conjunctivae became congested and a typical eye reaction reappeared. The longer the interval between the disappearance of the ophthalmic reaction and the subsequent injection of protein, the less frequently did this reappearance of the reaction take place. However, in one rabbit, under these conditions an eye reaction reappeared after an interval of 6 months.

The Development of Eye Reactivity Following Administration of the Intact Cell by Different Routes

The following experiment was carried out to determine whether eye reactivity results not only when injections of heat-killed pneumococci are made into the skin but also when the injections are made by other routes. Accordingly, several rabbits of one group were given repeated intravenous injections and those of another group intraperitoneal injections. The results of this experiment are summarized in Table III. It will be seen that in rabbits injected intravenously eye reactions were never obtained. Only two rabbits were given intraperitoneal injections, and neither gave an eye reaction when tested. It is appreciated, however, that the number in this instance is too small to allow a definite conclusion. Swift and Derick (4) have also shown in the case of hypersensitiveness to nonhemolytic streptococci, that the increased eye reactivity does not result from intravenous injections.

The Development of Eye Reactivity Following Repeated Injections of Soluble Derivatives of Pneumococcus

A study was made of the incidence of the eye reactivity in rabbits that have received repeated injections of solutions of various cell constituents. Rabbits were given intracutaneous or intravenous injections of (1) a solution of the bacteria prepared by freezing and thawing, (2) a solution of the "nucleoprotein" obtained by precipita-

tion of the bacterial solution by weak acid, (3) the supernatant fluid after precipitation of the "nucleoprotein," and (4) a solution of the bacterial cells resulting from autolysis. In Table III a summary is given of the results of this experiment. It is seen that in none of a total of 41 rabbits have eye reactions been observed after the animals had received repeated intracutaneous or intravenous injections of solutions derived from pneumococci.

TABLE III

The Incidence of the Eye Reaction in Rabbits Repeatedly Injected with Pneumococci or Their Products by Different Routes

Material administered	Route of administration	Number of rabbits	Result of eye test	
			Positive	Negative
Heat-killed Pneumococcus (R or S)	Intracutaneous	60	40	20
	Intravenous	91	0	91
	Intraperitoneal	2	0	2
Solutions	Intravenous	17	0	17
Nucleoprotein	Intracutaneous	8	0	8
	Intravenous	8	0	8
Solution (nucleoprotein removed)	Intracutaneous	4	0	4
Autolysates	Intravenous	6	0	6

The Development of Eye Reactivity Following Infection by Pneumococcus

The question naturally arises whether eye reactivity accompanies or follows infections by Pneumococcus. Rabbits were infected by injections of live cultures of Pneumococcus. Infection was induced by three different routes.

1. Infection by Skin.—Each of 18 rabbits received an intracutaneous injection of 0.2 cc. of an 8 to 10 hour culture of Type III Pneumococcus. All the animals developed a septicemia lasting from 1 to 7 days, and all but 2 eventually recovered from the infection. Viable organisms were recovered from the skin lesion only

during the first 2 to 4 days. About 3 weeks after infection had been induced, the animals were tested by the instillation into the conjunctival sac of "nucleoprotein" derived from a strain of Pneumococcus Type II. Five of the 16 rabbits gave positive eye reactions.

2. Infection by the Trachea.—Twelve rabbits received varying amounts of young broth cultures introduced intratracheally. Temperature variation in these animals indicated that infection occurred. All recovered. Sixteen days later eye tests were conducted in the usual manner. Of the 12 rabbits, 4 gave the ophthalmic reaction when tested in the usual way.

3. Infection by Spraying.—Seventeen rabbits survived infection by spraying with pneumococci according to the technique described by Stillman (5). These animals were tested for the presence of eye reactivity 2 to 3 weeks after the spraying. Of the 17 rabbits, 5 gave positive eye reactions. The data of the experiment are given in Table IV.

TABLE IV

The Incidence of the Eye Reaction in Rabbits Following Artificial Infection with Pneumococcus

Method of infection	Number of rabbits	Eye reaction	
		Positive	Negative
Skin	16	5	11
Intratracheal	12	4	8
Spraying	17	5	12

It is evident that eye reactions may be elicited in rabbits following experimental infection by Pneumococcus in the skin or trachea.

Relation between Eye Reactivity and Active Resistance to Infection

It has been pointed out in an earlier communication (6) that rabbits acquire an active resistance to infection following repeated intracutaneous injections of heat-killed pneumococci. It is now seen that rabbits so injected also may develop an eye reactivity to derivatives of Pneumococcus. On the other hand, rabbits receiving intravenous injections also acquire a resistance to infection, but they do not become eye reactive. Moreover, rabbits which have been given repeated injections of solutions derived from Pneumococcus acquire neither a resistance to infection (6), nor eye reactivity. While it may be concluded, therefore, that eye reactions do not necessarily accompany

active resistance to infection it is important to note that eye reactions were never observed in animals without resistance to infection.

Relation of Eye Reactivity to the Secondary Reaction

It has been previously reported (7) that the reaction at the site of injection following the first intracutaneous inoculation of heat-killed pneumococci in a rabbit usually disappears within 4 or 5 days. At about the tenth day, however, in certain animals there occurs a recrudescence. It seemed of interest to determine if possible whether the animals showing a secondary reaction were especially prone to develop eye hypersensitiveness. The data bearing on this point are summarized in Table V. It will be seen that a total of 23 rabbits were observed. Of 15 animals developing a secondary reaction, 12

TABLE V

Relation of the Ophthalmic Reaction to Secondary Skin Reaction

Total number of rabbits	Rabbits giving eye reaction	Rabbits not giving eye reaction
With secondary skin reaction, 15	12	3
Without secondary skin reaction, 8	3	5

gave a positive ophthalmic reaction, while 3 did not. Of 8 rabbits showing no secondary reaction, only 3 gave positive eye reactions. Although there is no absolute correlation between the development of eye sensitivity and the secondary reaction, nevertheless, eye reactions appear to occur with greater frequency in the rabbits which show the secondary reaction. Derick and Swift (4) also report the existence of a "rough parallelism" between the occurrence of eye reactions and the secondary reactions in rabbits injected with *Streptococcus viridans*.

DISCUSSION

The present communication records the development of an increased eye reactivity in certain rabbits which have previously received repeated intracutaneous injections of heat-killed pneumococci. The eye reactivity is manifested by a reaction, after scarification of the

cornea, to either the "nucleoprotein" or a solution of Pneumococcus from which the acid precipitable and heat coagulable proteins have been removed. The reaction, in the less severe form, consists of the injection of the conjunctival blood vessels; in the more severe forms, the cornea is also involved and exhibits a turbidity with frequently the formation of a pannus. Living R cells or the specific soluble substances of Pneumococcus do not elicit the eye reaction.

Unlike the skin reactivity reported previously, the eye reactivity does not occur in rabbits which have been given repeated intravenous injections of the heat-killed bacteria. Eye reactivity does not follow repeated intravenous or intracutaneous injections of rabbits with various solutions derived from the organism.

Increased eye reactivity was also found to be present in animals which had recovered from infection induced by injection of pneumococci into the skin or trachea, or by spraying. The eye reaction was elicited only in animals resistant to infection, but the relationship of the eye reactivity to active resistance still remains obscure. The eye reaction occurred more frequently in rabbits with a secondary reaction following the primary skin reaction to the first intracutaneous injection of bacteria than it did in animals not exhibiting a secondary reaction.

It may be of interest to point out that Clough (9) called attention to the "ophthalmo-reaction" in pneumonia patients. Without scarifying the cornea, he observed in 6 of 15 patients an eye reaction to one drop of 1 per cent Pneumococcus protein, while only 1 of 20 normal individuals gave a similar reaction.

SUMMARY AND CONCLUSIONS

1. About two-thirds of the rabbits injected intracutaneously with suspensions of heat-killed S or R pneumococci develop an increased eye reactivity.

2. Eye reactions in these animals may be elicited by the instillation of "nucleoprotein," or of a solution of Pneumococcus from which the acid precipitable and heat coagulable proteins have been removed. The eye reactions are not elicited, under the conditions described, by living R cells or the protein-free, type-specific, polysaccharides.

3. Rabbits do not develop an increased eye reactivity following intravenous injections of the intact cell.

4. Rabbits do not develop an increased eye reactivity following injections of soluble derivatives of Pneumococcus.

5. Experimental infection by Pneumococcus may stimulate eye reactivity in rabbits.

6. Eye reactivity occurs in animals actively resistant to infection.

7. Eye reactions are observed more frequently in rabbits which show the secondary reaction.

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REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

VI. Hypersensitiveness to Pneumococci and Their Products

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Plate 11

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In the preceding papers of this series, it has been shown that when rabbits are injected intracutaneously with a suspension of heat-killed pneumococci, a reaction occurs at the site of injection (1). With repeated injections, the reactions increase in size and intensity until four to six inoculations have been made, after which the reactions become decreased. Following the intracutaneous injections, the rabbits acquire a skin reactivity to certain derivatives of Pneumococcus, and in certain instances, also an eye reactivity to the same derivatives (2, 3). It was pointed out that while a state of skin reactivity may follow the injection of solutions of pneumococci as well as suspensions of the dead organisms and regardless of the route of administration, the state of eye reactivity arises only when intact cells have been injected intracutaneously, and less frequently following experimentally induced infection with Pneumococcus. Under either condition, it seems that a localization of the bacteria is essential in order that the animals develop eye reactivity.

It is well known that when rabbits that have received repeated injections of non-bacterial protein, and are later injected with small amounts of the same protein into the skin, marked reactions occur, the "Arthus reaction" (4). This skin sensitivity is now definitely known to be associated with the presence of specific antibodies in the circulating blood. It seems likely that the "skin reactivity" to pneumococci, and their protein derivatives, closely resembles the Arthus reaction, while the "eye reactivity" seems to depend upon

factors which are different from those operative in the phenomenon of "skin reactivity." It has seemed advisable therefore to compare all these reactions in a series of rabbits treated simultaneously.

EXPERIMENTAL

Each rabbit of three groups of eight received repeated injections of one of three different preparations. All the rabbits of the first group received injections of a solution of crystalline egg albumin; each of the second group received injections of a solution of "nucleoprotein" derived from a Type II (S) Pneumococcus; the rabbits of the third group received injections of a suspension of heat-killed Type I Pneumococcus. In four animals of each group the injections were made intracutaneously, in the other four the injections were made intravenously.

The injections were made once a week in quantities of 0.2 cc., which, in the case of the bacterial suspension, contained the organisms from 2 cc. of the original broth culture; while in the case of the protein solutions, 0.2 cc. of the one solution contained an amount of "nucleoprotein" of Pneumococcus estimated to be 0.6 mg., and 0.2 cc. of the egg albumin solution contained 3 mg. of the protein. The number of injections differed in the different animals, as will be brought out later. The animals were bled immediately preceding each injection, so that the development of circulating antibodies might be followed. Following each injection the character of the reaction was carefully recorded. At definite intervals, to be specified later, eye and skin tests were performed by the technique described in former communications (2, 3).

Repeated Injections of Rabbits with Crystalline Egg Albumin

a. Intracutaneous Injections.—Two rabbits were given 5 weekly injections of egg albumin in the skin and two animals received 10 similar injections. The earlier intracutaneous injections of egg albumin were not followed by definite local reactions. With later injections, there was always an extensive reaction at the site of injection. Thus following the first injections, only a pale erythematous blush was seen, and this disappeared within a day or two. In two rabbits, the fourth injection into the skin caused a definite reaction, which consisted of a central area of ecchymosis surrounded by a broad area of edema. The fifth injection was followed by a local reaction in all the rabbits. This reaction was typical of the Arthus phenomenon as already described by a number of workers (Arthus (4), Nicolle (5), Lewis (6), Opie (7), and others). The reaction following the sixth injection was perhaps more severe, but with subsequent injections the intensity of the skin reaction remained approximately the same. The more severe reactions began to appear within a few hours after the injection, and reached a maximum intensity in from 24 to 48 hours. At that time (Fig. 2) the size of the reactions varied from 4 to 6 cm. in diameter; they were circular, circumscribed and markedly elevated. The reactions contained a central area of ecchymosis and

necrosis which measured from 1 to 2.8 cm. The ecchymosis was surrounded by a thin, crimson band, 0.2 to 0.5 cm. wide. The remainder of the lesion was pink to red in color and consisted of a soft edematous ring. The edema usually disappeared within 3 to 5 days, and the area of ecchymosis was gradually transformed within a week into a black scab which persisted for 2 weeks or more.

A study of the precipitating antibodies present in the sera of these rabbits each week revealed that the precipitins increased gradually, the titre of the serum reaching 1:400,000 by the fifth injection. The titre continued to increase until the seventh injection when a maximum of 1:1,000,000 was reached, and it was maintained at that point during the time the injections were continued. The antibody content of the serum, therefore, rose concomitantly with the reactivity of the skin.

Twenty days after the final intracutaneous injection of egg albumin, the rabbits were tested for skin and eye hypersensitiveness. Skin tests were conducted by injecting into the skin different quantities of egg albumin. The dilutions employed contained from 3 to 0.015 mg., and the volume of fluid injected in each test was 0.2 cc. Skin tests were also performed on each rabbit with 0.2 cc. (0.6 mg.) of the Pneumococcus "nucleoprotein." There was some variation in the severity of the reactions to the smaller quantities of egg albumin, but the animals reacted locally with all the dilutions employed. The reactions were of the same character as those described above. No skin reactions were observed following the intracutaneous introduction of Pneumococcus protein.

Eye tests were done on the same day by the technique used in the preceding study (3). After scarifying the cornea, one drop of a solution of egg albumin was instilled into the conjunctival sac. The opposite eye was tested in a similar manner by instilling one drop of a Pneumococcus "nucleoprotein" solution. In none of the rabbits of this entire group were eye reactions elicited either by the solution of egg albumin or by the solution of "nucleoprotein" of Pneumococcus.

b. Intravenous Injections.—Four rabbits were given each week an intravenous injection of 0.2 cc. of a solution of egg albumin containing 3 mg. of the protein. Two rabbits received a total of 5 injections and two animals were given 10 inoculations.

The development of precipitating antibodies in the sera of these rabbits was similar to that observed in the rabbits which were given intracutaneous injections. The precipitin titre of the sera gradually increased to 1:1,000,000 at the seventh injection, and it remained at this point during the period of subsequent injections.

Twenty days after the last intravenous injection of egg albumin the animals were tested for skin and eye hypersensitiveness. The tests consisted of the inoculation of solutions of egg albumin and of Pneumococcus "nucleoprotein" into the skin and the separate instillation of these solutions into the conjunctival sacs in the same manner as described in testing the animals injected intracutaneously. Typical Arthus reactions in the skin were obtained in all the rabbits at the site of injection of the egg albumin solution, while no skin reactions occurred at the site

of injection of the Pneumococcus "nucleoprotein" solution. No eye reactions occurred in any of the animals tested by the instillation of either the egg albumin or the "nucleoprotein" solution. Derick and Swift (6) and Rhoads (9), also found that eye sensitivity did not occur in rabbits which gave typical Arthus reactions in the skin.

Repeated Injections of Rabbits with the "Nucleoprotein" of Pneumococcus

Eight rabbits were given repeated injections of a solution of "nucleoprotein" derived from a strain of Type II Pneumococcus. Injections of 0.2 cc. of the solution were made once a week. In four of the rabbits the injections were made intracutaneously and in the remainder, intravenously.

a. Intracutaneous Injections.—Of the four rabbits receiving intracutaneous inoculations, two received 5 injections and two, 10 injections. Following the earlier injections no visible local reactions occurred. Following the fourth injection all of the animals exhibited a reaction at the site of injection. At this time the reactions measured from 4 to 6 cm. in diameter. The skin showed slight, if any, elevation, but it was definitely thickened, and it assumed a pink color. With subsequent injections, the reactions increased in intensity until the seventh inoculation, and from then on the reactions remained of about the same intensity. The more severe reactions measured from 5 to 8 cm. in diameter. The skin was definitely elevated and was of a deep red color. A central area of ecchymosis frequently developed, but necrosis of the skin was never observed. The edema usually disappeared in 2 or 3 days, and within 5 to 6 days the lesion was completely resolved. These skin reactions to the "nucleoprotein" of Pneumococcus were strikingly similar to the Arthus reactions observed in rabbits hypersensitive to egg albumin, but they were never as severe as the latter reactions. This resemblance is seen in Figs. 1 and 2, which illustrate both skin reactions.

The development of circulating antibodies was estimated by determining each week the ability of the sera of these rabbits to cause agglutination of R pneumococci. A gradual increase in the agglutination titre occurred until the seventh injection, when a titre of 1:320 was reached, and the titre was maintained at that point during the remaining period of injection.

Twenty days after the last injection tests of skin and eye hypersensitiveness were made. The sensitivity of the skin was tested by the injection of 0.6 mg. (0.2 cc.) of the nucleoprotein of Pneumococcus and 3 mg. (0.2 cc.) of egg albumin. Eye tests were also performed with these proteins. In all the rabbits, skin reactions of the type already described occurred at the site of injection of the Pneumococcus "nucleoprotein" only. No reactions occurred at the site of injection of the solution of egg albumin. None of the rabbits exhibited eye reactions to either protein.

b. Intravenous Injections.—Four rabbits were given intravenous injections of a solution of Pneumococcus "nucleoprotein." Two rabbits received 5 injections and two received 10 injections. The development of antibodies in the sera of these

animals was studied, and it was found that the rate and time of appearance of anti-R agglutinins in these rabbits did not vary materially from the rate and time of appearance of those antibodies in the rabbits receiving intracutaneous injections of Pneumococcus "nucleoprotein" solution. The subsequent injections had no further effect on the antibody titre of the sera.

Twenty days after the last injection of the antigen, the animals were tested for skin hypersensitiveness by intracutaneous injections of solutions of Pneumococcus "nucleoprotein" and of egg albumin, and they were tested for eye hypersensitiveness by the separate instillation of solutions of these two proteins into the conjunctival sacs. The same quantities of the solution and the same methods were used as those employed in the previous experiment. Skin reactions occurred in all animals at the site of injection of the Pneumococcus "nucleoprotein." No skin reactions occurred at the site of injection of the egg albumin solution. The positive skin reactions resembled those which occurred in the animals which had previously been given repeated injections of Pneumococcus protein into the skin. No eye reactions occurred in any of the animals following the instillation of either the "nucleoprotein" solutions or the egg albumin solution.

Injection of Rabbits with Suspensions of Heat-Killed Pneumococcus Type I

Eight rabbits were given repeated weekly injections of suspensions of heat-killed Pneumococcus Type I; in four the injections were made into the skin and in four into a vein. At each injection 0.2 cc. of the suspension was given and this contained the bacteria from 2 cc. of broth culture.

a. Intracutaneous Injections.—Two rabbits received 5 injections and two received 10 injections. The reaction of the skin to successive injections of pneumococci has been described in detail in an earlier communication (1), but for purposes of comparison with the skin reactions to soluble proteins, however, they will be described again in brief. The first injection was followed locally by a sharply circumscribed, indurated, pink nodule which disappeared within a few days. A secondary reaction frequently followed the disappearance of the first reaction. With repeated inoculations, the reaction at the site of injection increased gradually in size, intensity, and duration until the fourth to the sixth injection. At this time, the skin was markedly elevated and assumed a reddish to purple hue. There was considerable edema and a central area of ecchymosis appeared at the site of which the skin frequently breaks down. The lesion of the severe reaction persisted for 3 to 5 weeks. The reaction following the injections after the fourth to the sixth had a modified character; they were flat, without edema or ecchymosis; they varied considerably in size, and they disappeared comparatively rapidly.

Weekly tests of the sera of these animals failed to show the presence of type-specific antibodies at any time and none of the sera possessed protective power for mice. Species-specific antibodies were detected as anti-R agglutinins for the first

time at the third bleeding. After this the titre of the serum for these agglutinins increased gradually and after the seventh injection an agglutination titre of 1:1280 was reached. The titre was maintained at that point during the remainder of the period of injections. Twenty days after the last injection of bacterial suspension these animals were tested for skin and eye hypersensitiveness. The injection of a solution of the "nucleoprotein" of Pneumococcus into the skin gave rise to a reaction at the site of injection in all animals and these reactions resembled the skin reaction to the "nucleoprotein" in the animals which had previously received repeated injections of the "nucleoprotein" solution. Reactions occurred in the eyes of three of the rabbits following the instillation of the solution of "nucleoprotein." In one animal no reaction occurred. The eye reactions involved the cornea but were without a pannus. (See Figs. 3 and 4.) These reactions have been described fully in a previous paper (3).

b. Intravenous Injections.—Four rabbits were given intravenous injections of heat-killed pneumococci Type I. The schedule of injections was the same as that employed for the intracutaneous inoculations. Two rabbits received 5 injections and two, 10 injections.

A study of the presence of antibodies in the sera of these rabbits showed that type-specific agglutinins were demonstrable after the second injection in a titre of 1:5 and 1:10. These antibodies increased and after the fourth injection the agglutination titre of the serum was 1:80. The titre was not influenced by further injections of the bacterial emulsion. Species-specific antibodies (R agglutinins) also appeared in the serum but the increase was more gradual. After the third injection, the titre of the serum for these antibodies was 1:10, and it increased to 1:640 after the fifth injection. From then on, the titre fluctuated slightly above and below that point.

Twenty days after the last injection of bacteria tests for skin and eye hypersensitiveness were conducted as described above. Injections into the skin of a solution of "nucleoprotein" of Pneumococcus gave rise to reactions at the site of injection in all the rabbits. These reactions could not be differentiated from those observed in the animals which were tested 20 days after repeated intracutaneous injections. Tests for eye hypersensitiveness all gave negative results.

The observations made in these experiments, in which the reactions occurring in three groups of rabbits treated in various ways were studied, may be summarized as follows:

With repeated intracutaneous injections of egg albumin, "nucleoprotein" of Pneumococcus or heat-killed Pneumococcus cells the rabbits all showed reactions at the site of injection. After each of the first 3 injections of egg albumin there occurred a faint erythematous blush. The reaction following the fourth injection was more severe, while the reactions following the fifth and sixth injections were very

severe, with necrosis of the skin and scab formation, the typical Arthus phenomenon. The reactions following the subsequent injections were approximately of the same intensity as those following the fifth and sixth injections.

When, instead of a solution of egg albumin, a solution of the "nucleoprotein" of Pneumococcus was repeatedly injected into the skin no reactions occurred after the first 3 injections. After the fourth injection a mild reaction occurred and after the fifth, sixth and seventh injections reactions of a considerable grade of severity were seen. These reactions also resembled the Arthus phenomenon, except that they were less severe. No necrosis of the skin was ever observed. The local reactions after the subsequent injections were of the same character and of the same intensity as those after the fifth and sixth injections.

When suspensions of bacterial cells were repeatedly injected into the skin, reactions at the site of injection occurred after the first and all subsequent injections. The reaction after the first injection was not of great severity and disappeared within 4 to 5 days. On about the tenth day, however, there occurred an exacerbation of the lesion at the same site, the so-called "secondary reaction." The intensity of the reactions which occurred following the subsequent injections became greater up to the fourth and sixth injections, after which the reactions became increasingly milder. No secondary reactions occurred except following the first injections. The superficial aspect of the skin reactions following the repeated injections of the suspensions of bacterial cells did not differ essentially from those of the reactions after the injections of egg albumin and "nucleoprotein" solutions, though in the former there has seemed less tendency for a zonal arrangement to be present, with ecchymosis in the center, about this erythema, and surrounding this an area of edema. But too much stress must not be laid on the difference in the superficial appearance of the different reactions. In the severe reactions after injection of suspensions of whole cells, necrosis and scab formation sometimes occurred just as they did after injections of egg albumin.

The reactions following the injections of whole cells do differ, however, from the reactions following repeated injections of egg albumin or "nucleoprotein" in that with the former every injection.

including the first, is followed by a reaction, while with the latter the reactions do not occur until 2 or 3 injections have been made. Moreover, following injections of egg albumin, or bacterial "nucleoprotein" when the reactions have become of maximum intensity, the reactions to all subsequent injections are also of maximum grade. On the other hand, when a reaction of maximum degree has followed one of the injections of suspensions of whole cells, the reactions to subsequent injections are milder and of a somewhat different character.

While, therefore, the skin reactions following injections of solutions of egg albumin, or bacterial "nucleoprotein," or of suspensions of pneumococcal cells, all resemble one another superficially and are like the Arthus phenomenon, the reactions following injections of the whole cell have certain characteristics, and others will be mentioned later, which indicate that they are of a different character. When the rabbits, which had received repeated weekly injections either intravenously or intracutaneously, of solutions of egg albumin, or solutions of Pneumococcus "nucleoprotein," or suspensions of heat-killed pneumococci, were tested 20 days after the last injection by injecting 0.2 cc. of a solution of egg albumin, or bacterial "nucleoprotein" intracutaneously, local skin reactions occurred in certain animals. In the animals which had been previously repeatedly injected intravenously or intracutaneously with egg albumin, typical Arthus reactions occurred when egg albumin solution was injected, but no reaction occurred when "nucleoprotein" solution was injected. Exactly the opposite results were obtained in the animals which had previously received injections of a solution of Pneumococcus "nucleoprotein." The reactions were therefore quite specific as concerns the proteins employed. In all cases these skin reactions were of a mild "Arthus" type. In the animals previously repeatedly injected, with suspensions of heat-killed pneumococci, intravenously or intracutaneously, no skin reactions were observed following the subsequent injection of egg albumin. When the tests were made with "nucleoprotein" solutions, however, definite skin reactions occurred and these did not differ materially from the reactions occurring in the tests previously mentioned.

Eye tests were performed on all the animals 20 days after the preparatory injections had been given. Into one eye a solution of egg albumin was instilled and into the other eye a solution of "nucleo-

protein" was instilled. In no cases were reactions observed except in animals in which previous repeated intracutaneous injections of a suspension of heat-killed pneumococci had been made. In three of these animals typical eye reactions (3) occurred, in one animal no change was observed.

The time of occurrence of all these reactions in the skin and eye was studied with especial reference to the development of antibodies in the blood. It will be seen by referring to the description of the experiment, that during the period when repeated injections of egg albumin and "nucleoprotein" were being made, either intracutaneously or intravenously, antibodies specific for the respective proteins gradually developed in the blood. They reached a maximum concentration about the seventh injection, and the titre of antibodies in the blood then remained at the maximum level during the remainder of the period of observation. The antibodies appearing in the serum coincident with the injections of Pneumococcus "nucleoprotein" were of the species-specific kind.

It will be observed that in the animals receiving repeated injections of egg albumin and "nucleoprotein" the development of antibodies in the blood ran a course parallel to the occurrence of the skin reactions following the repeated injections of the proteins. The concentration of antibodies continued at a high level and was high at a time when the subsequent skin tests were made, 20 days after the end of the series of injections. There seems therefore to be a definite relationship between the presence of antibodies in the blood and the occurrence of the skin reactions following injections of egg albumin and "nucleoprotein." This is also the case in the rabbits in which repeated intravenous injections of suspensions of heat-killed pneumococci were made. In this case type-specific as well as species-specific antibodies appeared in the blood. On the other hand, in the animals in which repeated intracutaneous injections of heat-killed pneumococci were made, only species-specific antibodies appeared in the blood, and the relation of the concentration of these antibodies in the blood to the occurrence of skin and eye reactions was not so definite. Antibodies did not appear in the blood until after the third injection and the maximum titre of the serum was not reached until after the seventh injection, following which the antibody concentration remained at the maximum level. Reactions in the skin,

however, occurred after the very first injections when no antibodies were present in the blood, and reached their maximum intensity after the fourth to sixth injections, before the concentration of antibodies in the serum had reached the maximum, and, in some cases, when the concentration of antibodies in the serum was still low. Moreover, the reactions following the later injections were modified in character and decreased in intensity and this at a time when the concentration of antibodies in the blood was still high.

The results of this experiment indicate in a more striking manner than do the previous studies that the skin and eye reactions which are observed following the intracutaneous injections of suspensions of whole cells are the manifestations of a different mechanism from that which is operative in the occurrence of skin reactions following the repeated injections of soluble proteins, or following the injection of the whole cells into the circulation. In the latter instance the reactions are associated with the presence of antibodies in the blood and are analogous to the Arthus phenomeonon; in the former case the type of skin and eye reactivity depends on some mechanism directly related to the localization of the bacteria in the skin, but the exact nature of this mechanism is still obscure.

This conclusion receives additional support from experiments undertaken to test the possibility of transferring the heightened "skin and eye reactivity" from animals which have received repeated injections of protein or bacteria to previously untreated animals.

That the Arthus variety of hypersensitiveness may be transferred to normal animals was first shown by Nicolle (5). This was more recently accomplished by Opie (6) who showed, in addition, that the severity of the skin reaction to the specific antigen, in rabbits injected with sera from actively sensitized rabbits, was approximately proportional to the concentration of antibodies present in the blood of the recipient animal after the transfer.

EXPERIMENTAL

Previously untreated rabbits were given intravenous injections of serum obtained from a single actively sensitized animal. The quantity of serum injected varied from 15 to 30 cc. On the day following the transfer of serum the recipient animals were bled in order to determine the titre of circulating antibodies and were then tested for the presence of skin and eye hypersensitiveness.

Transference of Hypersensitiveness Induced by Repeated Injections of Egg Albumin

Each of six normal rabbits was injected intravenously with 16 to 27 cc. of serum from one of six animals which gave intense reactions when a solution of egg albumin was injected into the skin. The donors had been injected repeatedly with egg albumin, three intravenously and three intracutaneously. On the following day the precipitin titre of the sera of the recipients varied from 1:10,000 to 1:50,000. Skin tests were performed in the usual manner. Five rabbits gave distinct skin reactions, with edema and, in one of the 5 reactions, a central zone of ecchymosis was present. In the sixth animal the skin reaction was doubtful, consisting only of a diffuse erythema. Eye tests were also done, but in each instance no reaction was obtained. Control of the specificity of the skin reaction was accomplished by intracutaneous injections of Pneumococcus nucleoprotein. No reactions followed the administration of this material.

Transference of Hypersensitiveness Induced by Repeated Injections of Pneumococcus "Nucleoprotein"

Each of eight normal rabbits was injected intravenously with 15 to 25 cc. of serum obtained from one of eight rabbits which had received injections of the "nucleoprotein" of Pneumococcus, four intravenously and four intracutaneously. On the following day the species-specific antibody titre of the serum of each of the recipients was measured by agglutination of R pneumococci. The titre was found to vary in the different animals from 1:20 to 1:80. The recipient animals were tested in the usual manner for eye and skin sensitivity to Pneumococcus "nucleoprotein." In six of the animals more or less intense skin reactions occurred, in one a doubtful reaction, and in the remaining animal no reaction was observed. The eye reactions were negative in each animal. Control of specificity was accomplished by skin and eye tests with a solution of egg albumin and no reactions were obtained to this material.

Transference of Hypersensitiveness Induced by Repeated Injections of Intact Pneumococci

Each of twenty rabbits was injected with 15 to 30 cc. of serum from one of twenty rabbits which had received repeated injections of a suspension of heat-killed pneumococci. Five of the donors received intravenous injections of S cells, and fifteen received intracutaneous inoculations of S pneumococci. It should be stated that eye sensitivity had been demonstrated in all of the donors which had received intracutaneous injections. On the following day in none of the recipient animals were anti-S agglutinins present in the sera, while the anti-R titre of the serum varied from 1:20 to 1:320. Skin tests were performed in the usual manner and all but one gave distinct and unmistakable reactions. Eye tests were done by

instillation of nucleoprotein in the conjunctival sac after scarification of the cornea. No eye reaction was elicited in any of the animals. This was to be expected in the recipient animals which received serum from donors which did not themselves exhibit eye sensitivity, but the lack of eye reactivity in the animals which had received intracutaneous injections and which had been shown to be "eye reactive" indicates that in these animals a special type of sensitivity was present, a type in which the sensitivity cannot be transferred by serum transfusion.

TABLE I

Reactions of Hypersensitiveness in Rabbits

Reactions	Following repeated injections of egg albumin		Following repeated injections of Pneumococcus protein		Following repeated injections of heat-killed pneumococci (Type I)	
	Intracutaneous	Intravenous	Intracutaneous	Intravenous	Intracutaneous	Intravenous
Reaction at site of each injection	1st 3 injec. − 4th–10th injec. +	×	1st 3 injec. − 4th–10th injec. +	×	1st injec. + increase in intensity until 4th–6th injec. Then decrease in reaction	×
Development of circulating antibodies						
(a) type-specific...	×	×	−	−	−	+
(b) species specific.	+	+	+	+	+	+
Active resistance.....	×	×	−	−	+	+
Increased sensitivity						
(a) skin.....	+	+	+	+	+	+
(b) eye.....	−	−	−	−	+	−
Transfer of sensitivity						
(a) skin.....	+	+	+	+	+	+
(b) eye.....	−	−	−	−	−	−

+ indicates presence of reaction.
− indicates absence of reaction.
× indicates tests were not done.

RÉSUMÉ

From the series of studies concerning the reactions of rabbits to injections of pneumococci and their products it appears that when heat-killed pneumococci or their products are repeatedly injected into rabbits, intravenously or intracutaneously, the rabbits become hypersensitive to subsequent intracutaneous injections of Pneumococcus proteins, as shown by a reaction in the skin, which is analogous to the Arthus phenomenon as exemplified in rabbits following injections of non-bacterial protein. The development of this hypersensitiveness occurs simultaneously with the development of species-specific antibodies in the blood, and this type of hypersensitiveness may be transferred to normal rabbits following the transfer of blood serum from the sensitive animals. The development of this type of sensitivity seems to bear no relation to active immunity, since animals repeatedly injected with soluble products of the pneumococcus cells show no active immunity and nevertheless are skin reactive.

When the whole, killed bacteria are injected repeatedly intracutaneously another type of hypersensitiveness, in addition to that just described, makes its appearance. This is shown by the fact that animals treated in this way react in a different manner to the individual injections of the bacteria into the skin. At the first injection there occurs a reaction in the skin, a primary reaction, which disappears after a few days, but is followed in about 10 days by a recrudescence. This recrudescence occurs even without a second injection, and is probably an evidence of the development of hypersensitiveness.

With the repeated intracutaneous injections of the whole organisms the skin reaction becomes gradually more severe; secondary reactions occur, however, only after the first injection. After 4 to 6 skin injections have been made the reactions become of lessened severity and are also modified in their character. The skin reactions during the course of injections, therefore, are probably different from unmodified Arthus reactions, though the Arthus type of sensitivity is coincidently present in the animals, as is shown by their exhibiting typical Arthus reactions when they are injected with small amounts of Pneumococcus protein 20 days later. That another type of sensitivity develops in these animals is also shown by the fact that they become eye sensitive

to Pneumococcus protein, while animals treated in other ways never do. Apparently when bacteria are localized in the skin there occurs the development of a new and different kind of hypersensitiveness.

SUMMARY AND CONCLUSIONS

1. Rabbits receiving either intracutaneous or intravenous injections of crystalline egg albumin acquire a "skin sensitivity" but not an "eye sensitivity" to the albumin.

2. Rabbits receiving either intracutaneous or intravenous injections of the "nucleoprotein" of Pneumococcus acquire a specific "skin sensitivity" to the "nucleoprotein" but not an "eye sensitivity."

3. Rabbits receiving intravenous injections of a suspension of heat-killed pneumococci acquire a "skin hypersensitiveness" but not an "eye hypersensitiveness" to the "nucleoprotein."

4. The skin hypersensitiveness to the respective proteins is associated with the presence of antibodies in the blood and is transferable by the injection of serum from an actively sensitized to a normal rabbit.

5. Rabbits receiving intracutaneous injections of a suspension of heat-killed pneumococci also acquire a "skin hypersensitiveness" to the "nucleoprotein" of the cell, but they may acquire an eye hypersensitiveness as well.

6. Rabbits injected intracutaneously with a suspension of heat-killed pneumococci show a primary and a secondary skin reaction following the first injection. The intensity of the reactions increases with subsequent injections up to the fourth to the sixth injection. With later injections these reactions change in character and decrease in intensity. These reactions bear no apparent relation to the presence of antibodies in the blood.

7. Rabbits injected intracutaneously with a suspension of heat-killed pneumococci develop "eye hypersensitiveness" which, under the experimental conditions, is not transferable from "eye reactive" to normal rabbits.

8. It appears, therefore, that following the injection of heat-killed pneumococci into the skin a special kind of "eye" and "skin hypersensitiveness" develops which is not related to the presence of circulating antibodies and which cannot be transferred from the sensitive to normal rabbits.

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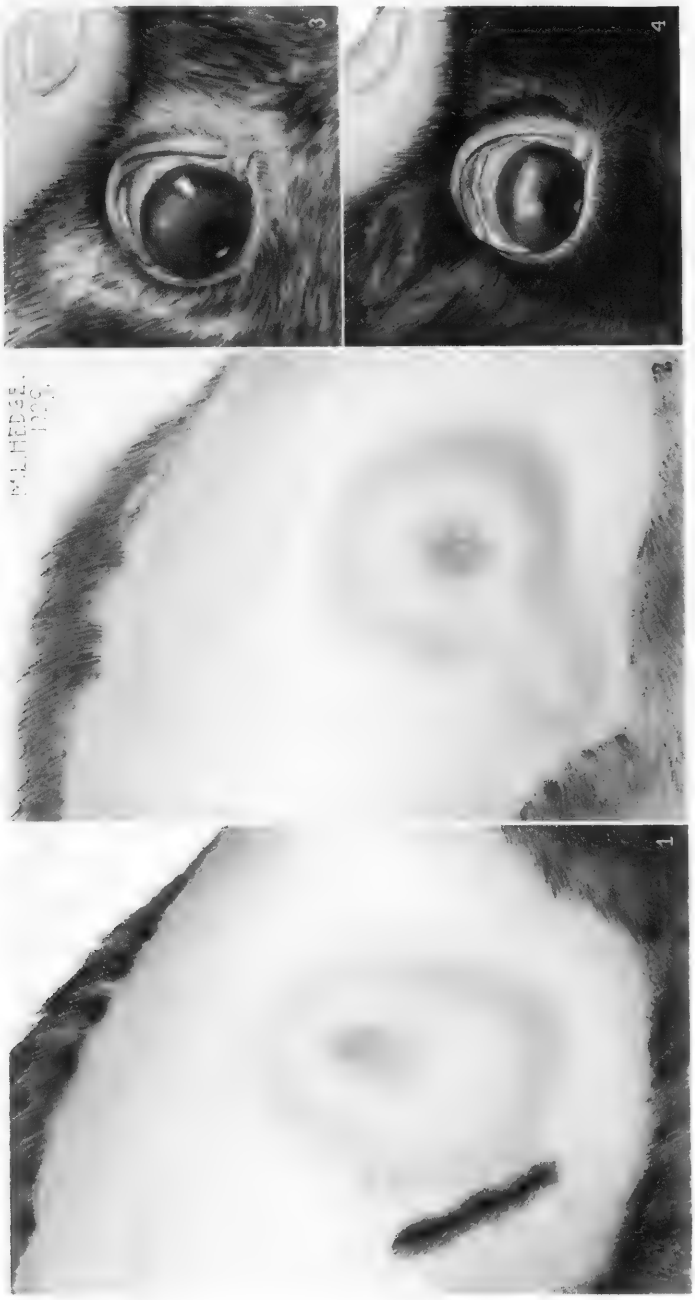
EXPLANATION OF PLATE 11

FIG. 1. Cutaneous reaction in a rabbit 24 hours after the injection of 0.6 mg. of the nucleoprotein of Pneumococcus. The rabbit had received preliminary injections of heat-killed pneumococci intravenously, and the last injection was made 20 days before the skin test.

FIG. 2. Cutaneous reaction in a rabbit 24 hours after the injection of 3 mg. of crystalline egg albumin. The rabbit had received preliminary injections of egg albumin intravenously, and the last injection was made 20 days before the skin test.

FIG. 3. Ophthalmic reaction in a rabbit which had been made hypersensitive by injections of heat-killed pneumococci into the skin. This illustrates the conjunctival reaction.

FIG. 4. Ophthalmic reaction in a rabbit which had been made hypersensitive by injections of heat-killed pneumococci into the skin. This illustrates the corneal reaction with the formation of a pannus.



(Julianelle: Intracutaneous injections of pneumococci. VI)

STUDIES ON LEUKEMIA IN MICE*

I. The Experimental Transmission of Leukemia

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In a strain of mice designated hereafter as C58, which has been inbred by brother-sister matings since 1921, it was observed that a considerable number of animals that lived more than 8 months had lymphatic leukemia.** The present cooperative investigation was undertaken early in 1928, since which time the mice have regularly been autopsied and the lesions examined microscopically.

In a preliminary report (1), we recorded the existence of this leukemic strain, and the fact that the leukemia is transmissible to other mice of the same strain by inoculation with tissue emulsions, at an earlier age than that at which leukemia occurs spontaneously.

LITERATURE

A complete account of the recorded attempts to produce or to reproduce the lesions of leukemia is not within the scope of this paper. Briefly, it may be stated that attempts to transmit leukemia from man to man, from man to lower animals, or from animals of one species to those of another, have been unsuccessful. These experiments have been reviewed recently by Opie (2).

The first and most widely known transmissible leukemia is that which occurs spontaneously in the fowl, and which Ellermann and Bang (3) and later Hirschfeld and Jacoby (4) and Schmeisser (5) transmitted to other fowl by inoculation with organ emulsions. Ellermann and Bang also transmitted the disease by inoculation

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** The diagnosis was first made by Dr. Alwin M. Pappenheimer in 1926.

with cell free filtrates. Both lymphoid and myeloid leukemia were thus transmitted, and change of type was noted in some of Ellermann's experiments. Fowl paralysis (neurolymphomatosis gallinarum), although not identical with leukemia, has points in common, and is transmissible (6).

The first report of successful transmission of mammalian leukemia is that of Snijders (7), who inoculated guinea pigs with tissue emulsions of a guinea pig which had spontaneous leukemia. The lesions produced were comparable to those of the spontaneous case, and were transmissible by subsequent transfers. Not only were typical leukemias encountered, but also cases of aleukemic lymphadenosis, leukosarcomatosis, and several almost pure tumors. Snijders considered his results as evidence favoring the neoplastic theory of leukemia. Mettam (8) has found conditions resembling leukemia in cattle (East coast fever and snotsiekte) to be transmissible.

In mice both lymphoid and myeloid leukemia have been observed to occur spontaneously, notably by Tyzzer (9), Haaland (10), Levaditi (11), Slye (12), Simonds (13), and Hill (14). Tyzzer and Haaland attempted to transmit the disease by inoculation, but without success.

Since we reported the transmission of leukemia in Strain C58, Korteweg (15) has recorded the transmission of "leukosarcomatosis" in mice. Korteweg's results are very similar to our own and, as will be seen, the "leukosarcomatoses" and "leukemias" which occurred in the course of our experiments are interpreted as varieties of the same disease. Korteweg found leukosarcomatosis to behave, on transfer, like a true tumor. His material differs from ours, however, in that the mice used for inoculation were not from a single strain, and were thus very different genetically. Furthermore, all of his material came originally from the tumor of a single mouse.

Spontaneous Leukemia in Strain C58

The cases of lymphatic leukemia and related conditions which have been observed in Strain C58 are similar in their anatomical and microscopical features to those reported by other observers. The varieties observed by Simonds (13) in the Slye stock have also been found in Strain C58. It will be necessary, however, to note certain general features of the disease as it has occurred in this strain.

Incidence.—A large proportion of mice of both sexes have had leukemia. Of 155 mice that died since May, 1928, 137 had leukemia. This, however, is not a final result as many mice are still alive. There are wide variations in the age of incidence and some animals have died at relatively early ages. The figures should not, therefore, be read in terms of percentage. An account of the history of leukemia in Strain C58 will be published in a subsequent report.

Age Incidence.—In 137 cases of spontaneous leukemia that died since May, 1928, the age at death is given in Table I. The youngest animal in this series was 186 days old. 104 or 76 per cent of the cases occurred between the 8th and 12th months; 18 or 13 per cent were less than 8 months old, and 15 or 11 per cent were over 1 year.

During the same interval, 18 other mice died and 4 were killed. Of these, 6 were diagnosed as "doubtful" (usually because of extreme post-mortem decomposition), and 16 as "not leukemia."

Occurrence of Other Diseases.—In the early history of the strain, many animals were discarded before the age at which neoplasms are commonly found. Since March, 1928, at which time discarding of animals was discontinued and necropsies regularly performed, a few neoplasms have been found other than those of the leukemic group under discussion. These include one case of epithelioma of the skin,

TABLE I

	Age in months													
	5	7	8	9	10	11	12	13	14	15	16	17	18	19
No. of animals	5	13	19	35	24	26	5	2	2	2	2	1	0	1

one of osteogenic sarcoma and two of carcinoma of the lung, all of which were associated with leukemia. There were also one case of chondro-osteosarcoma and one salivary gland tumor. Of other diseases, none has been constant. External parasites have occasionally appeared in the colony, and a few cestodes have been found.

Diagnosis.—The final diagnosis has, in each case, been made by gross and microscopic examination of the organs at autopsy. In typical cases the lesions are sufficiently characteristic to enable accurate diagnoses to be made by gross findings alone, although sections have been regularly examined. In only two cases were diagnoses of leukemia made at autopsy changed after examinations of the sections, but a few unsuspected cases of leukemia in early stages were found microscopically.

It is convenient to recognize the disease during life, and this we have found possible in a very large number of cases. In general, en-

largement of the spleen is the first symptom noted, and can be recognized by palpation of its lower free end and usually distinct edges. The number of cases detected in early stages has been roughly proportional to the regularity of periodic examinations of spleens in the colony. Although splenomegaly is also found in conditions other than leukemia, we have encountered but few such instances in these mice. There is also emaciation, causing the pelvic bones to become prominent, the nose sharp, and the vertebrae of the tail distinct. The abdomen becomes distended and feels firm and heavy in spite of the emaciation. The animal is less active and its movements slow. The presence of large intrathoracic tumors such as occur in the "leukosarcomatoses" has frequently been diagnosed by the occurrence of labored

TABLE II

Leucocyte Counts on 250 Normal Mice of Strain C58

White cell count	No. of counts
5–10,000 per cmm.	81
10–15,000 " "	110
15–20,000 " "	64
20–25,000 " "	27
25–30,000 " "	5
30–35,000 " "	2
35–40,000 " "	1

breathing. Blood examinations often give valuable confirmatory evidence of the presence of leukemia, but the absence of a typical blood picture does not necessarily indicate the absence of leukemic changes in the tissues. The data in Table II of leucocyte counts in mice of Strain C58, compiled from 290 counts on 250 normal, uninoculated mice between the ages of 1 and 4 months may be used for comparison with the counts on cases of spontaneous and transmitted leukemia.

All of these mice were subsequently used for inoculation. From the table it will be seen that in only 3 of the 290 examinations were there more than 30,000 white blood cells per cubic millimeter. Blood smears were not examined in every instance, but neither in those which were examined nor in the counting chamber when the leucocytes were counted were abnormal cells observed. In addition, smears of the

blood of 175 mice less than 6 months of age, which were made in the spring of 1928, failed to show abnormal cells resembling those in the typical leukemic cases. Many of these animals later developed leukemia spontaneously.

In 131 cases of spontaneous leukemia, the distribution of the leucocyte count is given in Table III, the highest count for each mouse being used.

Of these, 6 were over 300,000 and 2 over 600,000. Thus in 22.9 per cent of these cases, the leucocyte count was never found to be above 30,000 per cubic millimeter, a figure occasionally reached in normal mice. In several cases with counts below 30,000, abnormal cells were seen in smears or in the counting chamber.

Of the 100 mice that at some time had leucocyte counts of 30,000

TABLE III

Leucocyte Counts in Spontaneous Leukemia

White cell count	No. of animals
under 30,000 per cmm.	31
30–50,000 " "	35
50–100,000 " "	35
over 100,000 " "	30

or more, 33 subsequently had diminution of the leucocytes, which in 18 cases were then less than 30,000. A few of these changes are, for example: from 66,000 to 11,000; 75,000 to 25,000; 354,000 to 31,900; 184,000 to 60,000; 229,000 to 3800 with subsequent rise to 46,000.

The abnormal cells which appear in the blood in typical cases of leukemia are larger than the normal mouse lymphocyte. The nuclei are large, usually round, and leptochromatic. When stained by one of the Romanowsky methods a nuclear network of chromatin is demonstrated, which is somewhat less dense than in the normal lymphocytes, though in both of these types of cells the structure is slightly more coarse than in the corresponding human leucocytes. Several nucleoli may be present, but are not constant. The cytoplasm is basophilic, frequently with small clear areas near the nucleus, or with small vacuoles. Specific granules are not present, but azure

granules are occasionally found. Mitoses have been observed. Many cells are intermediate in type between these immature forms and the normal lymphocytes.

Course.—No instance of recovery from spontaneous leukemia has been observed in this strain. The disease is progressive, but variations have been observed in the size of enlarged lymph nodes. There is increasing enlargement of the spleen, weakness, emaciation, and frequently marked anemia. In several cases the course was protracted, lasting in one instance 10 months after enlargement of the spleen was first noticed.

Varieties of Manifestation, and Terminology.—It is known that both myelogenous and lymphatic leukemia occur in mice, as well as related lymphoid hyperplastic and neoplastic conditions, which offer difficulties in classification. Some of the latter correspond to the "pseudoleukemias," "leukosarcomatoses" and related conditions as observed in man. Hodgkin's disease appears to be an exception, for but five cases have been reported in mice (13, 16).

In Strain C58 the cases of leukemia which have been examined since the spring of 1928 have been only of the lymphatic type. Among these are mice showing the typical lesions of lymphatic leukemia including a characteristic blood picture, and also, in smaller numbers, those which would correspond to cases of "leukosarcomatosis" or "pseudoleukemia," as described by Simonds (13). In all cases, however, the type of cell comprising the infiltrations is of lymphoid nature. The similarity of these infiltrations, the variations in the blood picture and the occurrence of cases intermediate in type between leukemia and leukosarcomatosis or lymphosarcoma, give one the impression that these different anatomical varieties are but variations in the distribution of lesions rather than differences of fundamental nature.

The distinction between "leukemia" and "pseudoleukemia," the latter term referring to Cohnheim's designation of that condition in which the gross and microscopic lesions are characteristic of leukemia, but in which the blood picture does not show leukemic changes, should not, in our opinion, be made in mice of this strain. Repeated blood examinations in mice with palpably enlarged spleens have shown that:

(1). Splenic enlargement frequently precedes the appearance of a leukemic blood picture;

(2) A leukemic blood picture usually occurs at some time during the course of the disease;

(3) The blood changes may be a very late manifestation;

(4) A leukemic blood picture is not always permanent, but may approach or reach normal without recovery or corresponding clinical changes.

In view of these variations in the blood picture, it is evident that the classification of a particular case as "leukemia" or "pseudoleukemia" depends on the stage at which death occurred, and that the blood picture is an inconstant symptom rather than an indication of fundamental differences. Likewise there are cases with lesions the distribution of which is intermediate between the diffuse changes of leukemia and the local growths of "leukosarcomatosis" or "lymphosarcoma." We therefore prefer to classify in one group all of those conditions which we believe to be fundamentally of the same nature. For this group the term "leukemia" is selected, not because it is literally descriptive, but because it is widely used and generally understood. "Pseudoleukemia," "leukosarcomatosis" and similar terms represent, as far as these mice are concerned, unimportant though interesting variations in the extent or distribution of the lesions.

Experimental Transmission of Leukemia in Strain C58

The first of our experiments on the transmissibility of leukemia in this strain was performed as follows:

A female mouse (50420) not previously used in any experiment was first observed to have a large spleen at the age of 7½ months. At 10 months the spleen was larger, and the circulating white blood cells numbered 694,000 per cubic millimeter, of which the great majority were lymphoid cells of immature type. The mouse was killed, the spleen removed aseptically, and a portion of it minced in sterile saline. Examination of the organs at autopsy showed enlargement of the spleen and liver and of the cervical, axillary, inguinal and mesenteric lymph nodes. The diagnosis of lymphatic leukemia was later confirmed microscopically.

The saline emulsion of spleen was immediately inoculated intraperitoneally into 8 mice of Strain C58 which were 4 weeks of age.

Following the inoculation, the leucocytes were counted at intervals and were found in each case to increase in number, reaching figures higher than are found in many cases of leukemia. The highest count found in this experiment was 262,000 about 3 weeks after inoculation. The increase in the number of white cells

appeared to be due to the presence in the blood stream of immature lymphocytes comparable to those of the spontaneous case.

Two of the 8 mice were killed at 17 and 21 days respectively after inoculation, while the rest were allowed to live until death occurred spontaneously in 32 to 35 days after inoculation. The gross appearance of Mouse 53983 shows the type of lesion found in this particular experiment, though it is not characteristic of all:

The spleen measured 1.9 x 0.5 x 0.3 cm. It was of normal color, the follicles were distinct but not larger than normal.

At the site of inoculation in the anterior abdominal wall was a tumor about 1 x 0.8 cm. in diameter. Near it and on the opposite side of the abdomen, were smaller nodules in the abdominal wall. The cervical nodes were enlarged, slightly greater on the right side. Axillary and inguinal nodes were but slightly enlarged. In the mesentery were many nodules, each less than 0.2 cm. in diameter. A mass of lymphoid tissue was behind the pyloric portion of the stomach, between it and the liver. There was also infiltration of the perirenal fat, forming tumor-like masses on either side of the vertebral column, mesial to the kidneys.

The liver, lungs, kidneys, thymus and mediastinal lymph nodes were normal.

Microscopic examination showed the lesions to be composed of lymphoid cells of abnormal type, with many mitoses. Smears of the tissues show that these are the same type of cell described above in the blood of leukemic mice.

It is significant that in this and subsequent experiments, the lesions of leukemia were obtained in mice dying of the disease at an earlier age than that at which any mouse has been observed to have leukemia spontaneously, either in this strain or in any other recorded in the literature.

From one of the animals in this experiment, the spleen was removed aseptically, a saline emulsion prepared, and other mice of Strain C58* inoculated. In this manner, by the inoculation of tissue emulsions** the disease has been repeatedly transferred, and is now in its 30th passage. The experimental leukemia of this series, originating from Mouse 50420 is designated as "Line A," the first transfer as A1, the second as A2, etc.

* We have not been successful in transmitting the disease to mice of other strains.

** Our usual procedure has been to inoculate intraperitoneally with emulsion of spleen or lymph node. Positive results have also been obtained after the inoculation of ascitic fluid, which in some experiments has been abundant, with heart-blood and with liver. Subcutaneous inoculation is also successful, but the distribution of the lesions is somewhat different.

The experiment was repeated, using tissues of other mice with spontaneous leukemia, and the experimental leukemias thus obtained are designated as "Line B," "Line C," etc. In all, 10 spontaneous cases have been used as donors, with successful transmission in each. Some of these lines have been allowed to die out. Four lines are now used for continued transmission and the current experiment in each is A30, E10, H10 and I9.

The lesions found in Experiment A1 are fundamentally the same as those of subsequent experiments, but variations have been observed in the distribution of the lesions, the degree to which individual organs are involved, and the occurrence of leukemic blood pictures. These variations are comparable to similar differences in distribution of the lesions observed in the spontaneous cases. In each of the experimental lines now being studied, minor differences in the distribution of the lesions, the type of reaction and the interval between inoculation and death are observed which, though not perfectly constant, are sufficiently characteristic to indicate the presence of differences in the inocula. Thus, after intraperitoneal inoculation, Line A is now characterized by the nearly constant presence of ascites; Line E by the relatively frequent occurrence of chest tumors with fluid in the pleural cavity, but infrequent ascites; Line H by somewhat higher leucocyte counts and marked infiltrations in the liver, without chest tumors and only rarely with ascites; and Line I by marked infiltrations in the kidneys and liver, with high leucocyte counts, but without chest tumors or ascites.

In the later transfers of each experimental line there has been a decrease in the average interval between inoculation and death, and, at the same time, less variation among animals inoculated with the same material. This enables us to obtain, at present, fairly uniform results after inoculation, with each experimental line presenting its individual characteristics. An abstract of these results is appended.

Microscopically the lesions found in different experiments are similar, in that they all consist of the presence of numerous large cells of lymphoid type with frequent mitoses. These cells appear in the blood stream and infiltrate distant organs or tissues. In some experiments the cells have infiltrated the liver, spleen, kidneys and pancreas, producing the same picture as in spontaneous leukemia. In

Experiment	No. of mice	Positive results	Negative results	Average period of survival (days)*
		Line A		
A1	8	8	0	33.5 (6)
A2	4	1	3	(a)
A3	25	20	5	28.19 (16)
A4a (b)	8	8	0	19.3 (6)
A4b	8	8	0	21.6 (7)
A4c	6	5	1	30.25 (4)
A5a (c)	6	6	0	12.6 (5)
A5b (c)	3	3	0	23.0 (2)
A6a (d)	4	4	0	16.25 (4)
A6b (e)	4	4	0	16.7 (3)
A6c (e)	4	3	1	22.0 (2)
A7a (f)	4	4	0	15.5 (4)
A7b (g)	4	4	0	20.3 (3)
A8	4	4	0	13.7 (3)
A9	4	2	2	(h)
A10	4	4 (i)	0	29.5 (2)
A11	4	2	2 (j)	20.0 (1)
A12	4	4	0	10.5 (2)
A13a	4	4	0	9.0 (3)
A13b	4	4	0	10.0 (4)
A14 (k)	8	8	0	10.8 (7)
A15	8	8	0	12.0 (7)
A16	4	4	0	15.7 (3)
A17	4	4	0	10.0 (3)
A18	8	8	0	9.4 (7)

* The figures in parentheses indicate the number of positive cases from which the average is calculated. Animals that were killed are not included.

(a) The one positive case was killed 46 days after inoculation. One of the negatives died at 4 days.

(b) Three mice of A3 were killed and used as donors, hence the subdivisions of A4. Similarly for A5, A6, A7 and A13.

(c) Donor from A4a.

(d) Donor from A5a.

(e) Donor from A5b.

(f) Donor from A6b.

(g) Donor from A6c.

(h) One killed at 15 days was positive. One died at 141 days (age 206 days), may be a spontaneous case.

(i) One died at 147 days (age 214 days) may be a spontaneous case. Not counted in average duration.

(j) One died 5 days after inoculation with negative result.

(k) Donor from A13a.

Experiment	No. of mice	Positive results	Negative results	Average period of survival (days)
Line A—Concluded				
A19	12	10	2	13.4 (9)
A20	12	12	0	10.5 (11)
A21	12	12	0	10.5 (11)
A22	12	12	0	13.0 (11)
A23	8	8	0	13.0 (7)
A24	5	5	0	7.25 (4)
A25	8	7	1 (l)	11.6 (5)
A26	8	6	2	10.75 (5)
A27	8	8	0	13.7 (7)
A28	6	6	0	20.0 (4)
A29	8	8	0	14.1 (6)
A30	4	4	0	9.0 (2)
Line B				
B1	12	5	7 (m)	54.5 (4)
B2	8	8	0 (n)	12.7 (6)
B3	8	2	6 (o)	17.0 (1)
B4	5	5 (p)	0	9.0 (3)
		Discontinued		
Line C				
C1	7	6	1	58.1 (6)
		Discontinued		
Line D				
D1	12	3	9	28.0 (2)
D2	5	4	1	17.3 (3)
D3	6	6	0	18.3 (6)
		Discontinued		

(l) The negative died three days after inoculation.

(m) Four negatives died in less than 4 days.

(n) In one case diagnosis is not certain. Nodes and spleen were enlarged, but animal was decomposed and partly eaten by other mice.

(o) The 6 negatives died 1 day after inoculation.

(p) One case doubtful because of post mortem decomposition.

Experiment	No. of mice	Positive results	Negative results	Average period of survival (days)
		Line E		
E1	9	8	1 (q)	66.8 (6)
E2	8	7	1 (r)	57.3 (6)
E3a	25	9	16	36.3 (8)
E3b	6	6	0	50.7 (6)
E4 (s)	4	4	0	51.3 (3)
E5	4	4	0	32.3 (3)
E6	4	4	0	25.0 (3)
E7	8	8	0	22.0 (7)
E8	8	8	0	26.8 (7)
E9	6	6	0	21.6 (5)
E10	8	8	0	19.5 (7)
		Line F		
F1	5	4	1	15.3 (3)
F2	3	3	0	21.0 (3)
		Discontinued		
		Line G		
G1	8	8	0	48.25 (8)
		Discontinued		
		Line H		
H1	2	1	1 (t)	35.0 (1)
H2	6	6	0	30.5 (4)
H3	4	3	1 (u)	20.5 (2)
H4	7	7	0	16.1 (6)
H5	4	4	0	17.7 (3)
H6	4	4	0	18.7 (3)
H7	8	8	0	15.0 (7)
H8	8	8	0	14.0 (7)
H9	6	6	0	17.5 (5)
H10	6	6	0	18.6 (5)

(q) The negative case died 36 days after inoculation. Others became ill not less than 49 days. Two killed at 109 and 121 days were positive.

(r) Negative case died 1 day after inoculation.

(s) Donor from E3a.

(t) The negative case had a large spleen but was too much decomposed to diagnose microscopically.

(u) The negative case died 2 days after inoculation.

Experiment	No. of mice	Positive results	Negative results	Average period of survival (days)
		Line I		
I1	4	4	0	55.0 (2)
I2a	4	1	3	(v)
I2b	6	6	0	29.0 (6)
I3	4	4	0	36.0 (3)
I4	4	4	0	20.0 (3)
I5	6	6	0	29.6 (5)
I6	8	8	0	25.7 (7)
I7	6	6	0	19.8 (5)
I8	6	6	0	13.4 (5)
I9	6	6	0	10.75 (5)
		Line J		
		(Inoculated at Cold Spring Harbor)		
J1	4	3	1	22.0 (3)
		Discontinued		

(v) The one positive result was in an animal killed 14 days after inoculation and used as donor for I3. Emulsion kept on ice 48 hours before use. Two animals died 4 days after inoculation.

other cases the infiltration of these organs may be minimal or absent, yet the mesentery and omentum may have numerous small nodules, the fat tissue of the abdomen may be infiltrated and ascites may be present. These lesions will be reported in detail in a separate paper.

The appended protocols include only those mice of Strain C58 which were inoculated with emulsions of tissues not subjected to any preliminary treatment other than mincing in saline, with the exception of Experiment I3, the material for which was kept in a refrigerator for 48 hours before use. In calculating the average period of survival, only those mice that died of transmitted leukemia are included. The figure in parenthesis after each average indicates the number of mice from which the average is calculated. Except where noted, the inoculations were made in New York.

In addition to these C58 animals, there were inoculated 107 mice from the Storrs-Little strain, 19 of Dilute Brown, 16 Bagg albinos, and 3 of Strain 85, all with negative results.

A series of experiments performed in the laboratory at Cold Spring Harbor has given results similar to the above obtained in New York. A mouse from Experiment A17 and one from A25 were sent to Cold Spring Harbor and used as the donors in subsequent experiments, and the series of transmissions obtained therefrom correspond to those in this line reported in the protocols.

Of 78 mice thus inoculated, 73 developed leukemia, and 5 died in less than 4 days after inoculation.

SUMMARY

Lymphatic leukemia has occurred with great frequency in a particular strain of mice which have been inbred by brother-sister matings since 1921. In addition to typical cases of leukemia are others which, because of the absence of leukemic changes in the blood, correspond to "pseudoleukemia" and others which, by the presence of unusually great enlargement of certain lymph node groups resemble the "leukosarcomatoses" as observed in man.

Examinations of the blood of leukemic mice have shown that leukemic blood pictures are not necessarily early in their appearance, nor are they constant. The blood picture may not, therefore, be used as a criterion for the separation of the two diseases (leukemia and pseudoleukemia) but merely indicates different phases of the same condition. Likewise, cases with lesions intermediate between the local growths of "leukosarcomatosis" and the more general lymphatic enlargements of leukemia suggest that these conditions differ only in the distribution of lesions but not in their nature.

Lymphatic leukemia occurring spontaneously in this strain may be transmitted to other mice of the same strain, and carried, apparently, for an unlimited number of transfers in animals at an earlier age than that at which leukemia occurs spontaneously. In each of 10 such experiments transmissions were obtained. The lesions produced by inoculation correspond to those of spontaneous cases, in that they consist of growths of abnormal lymphoid cells which infiltrate tissues and organs and often appear in the circulating blood. Only minor differences have occurred, some of which are characteristic of certain experimental lines. After repeated transfers, the disease tends to run a more acute course.

Among the cases in which transmissions occurred, are some without leukemic changes in the blood, and many with local growths at the site of inoculation or in certain node groups. The differences in the blood pictures and distribution of lesions (which latter may be influenced to some extent by the method of inoculation) correspond to similar differences which are sometimes observed in the spontaneous cases.

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